Maternal gastrointestinal nematode infection alters hippocampal neuroimmunity, enhances long-term potentiation and spatial memory and improves resistance to direct infection in mouse offspring

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Abstract

Maternal factors that induce neuroinflammation in the developing brain can be detrimental. Gastrointestinal (GI) nematodes, in contrast, induce immunoregulatory responses, and may be beneficial. The goals of this research were to determine the impact of maternal infection with the murine GI nematode, *Heligmosomoides bakeri*, on offspring spatial memory, hippocampal long-term potentiation (LTP), the neuroimmune environment, and resistance to direct infection.

Two spatial memory tests showed an enhancement of spatial memory in response to maternal *H. bakeri* infection in both male and female pups. At post-natal day (PD) 17, pups of infected dams retained object location memories for three hours in the Object Location Test, and at PD 34, they retained their ability to find an escape location in the Barnes Maze Test for one week, whereas pups of uninfected mothers did not. These novel findings indicated that a maternal GI nematode infection positively influenced the spatial memory of uninfected juvenile offspring.

As hippocampal LTP is an important process for spatial memory formation, acute hippocampal slices from PD 21-24 male pups were used to record field excitatory postsynaptic potentials in the CA1 region evoked by Schaffer collateral stimulation. LTP was enhanced in response to maternal infection. This was consistent with hippocampal gene expression data from RNA-seq analysis that indicated accelerated development of mature glutamate synapses at PD 23 in male and female pups of infected dams. These findings explain, at least in part, the enhanced spatial memory in juvenile pups of *H. bakeri* infected dams.

In response to an *H. bakeri* infection, infected mothers mount a strong Th2/Treg immune response. As the maternal immune system influences the offspring immune system that, in turn, can strongly influence neurodevelopment and behaviour, the impact of maternal infection on the ability of juvenile male and female offspring to resist direct infection was assessed as an index of peripheral immunity, and the hippocampal neuroimmune environment of pups was also explored. At PD 27, offspring were directly infected with *H. bakeri*. One month later, offspring of infected mothers were more resistant to infection as evidenced by lower worm burdens and

fecundity, indicating functional protection against direct *H. bakeri* infection, consistent with the maternal transfer of *H. bakeri*-specific IgG1. At PD 22, the neuroimmune environment was also altered by maternal infection. Immunohistochemistry revealed greater numbers of microglia and astrocytes, and a greater percentage of microglia expressing the CD206 marker that is typically up-regulated in response to Th2 cytokines. Also, hippocampal gene expression data showed up-regulation of the TGF-β signaling pathway associated with immunoregulation. These latter results indicate that the neuroimmune environment in juvenile uninfected pups mimicked the peripheral immune environment of their infected mother.

The final study determined if the enhanced spatial memory of juvenile male and female offspring of *H. bakeri* infected dams was still evident at adulthood. The Barnes Maze Test revealed that, in response to maternal infection, three-month old uninfected female offspring had enhanced spatial memory, but males did not. This difference between sexes may in part be explained by the influence of sex hormones on the immune system.

Together, these original findings demonstrate that a maternal GI nematode infection during pregnancy and lactation enhances spatial memory in uninfected offspring. Evidence suggests a potential causal pathway to explain this, whereby the Th2/Treg immune response of the infected mother is mimicked in the uninfected pup brain, driving enhanced hippocampal LTP that in turn promotes spatial memory. These findings shed light on a possible unappreciated benefit of maternal GI nematode infection and highlight a potential increase in offspring fitness.

Résumé

Les facteurs maternels qui induisent une neuroinflammation dans le cerveau en développement peuvent être préjudiciables. Les nématodes gastro-intestinaux, en revanche, induisent des réponses immunorégulatrices et peuvent être bénéfiques. Les objectifs de cette recherche étaient de déterminer l'impact de l'infection maternelle par le nématode gastro-intestinal murin, *Heligmosomoides bakeri*, sur la mémoire spatiale de la progéniture, la potentialisation à long terme (PLT) de l'hippocampe, l'environnement neuro-immunitaire et la résistance à l'infection directe.

Deux tests de mémoire spatiale ont montré une amélioration de la mémoire spatiale en réponse à l'infection maternelle par *H. bakeri* chez les petits mâles et femelles. Au 17e jour postnatal, les petits des mères infectées ont conservé la mémoire de la localisation des objets pendant trois heures dans le test de localisation des objets, et au 34e jour postnatal, ils ont conservé leur capacité à trouver un lieu de fuite dans le test du labyrinthe de Barnes pendant une semaine, alors que les petits des mères non infectées ne l'ont pas conservée. Ces nouveaux résultats indiquent qu'une infection maternelle par un nématode gastro-intestinal a une influence positive sur la mémoire spatiale de la progéniture juvénile non infectée.

La PLT hippocampique étant un processus important pour la formation de la mémoire spatiale, des tranches aiguës d'hippocampe provenant de chiots mâles PD 21-24 ont été utilisées pour enregistrer des potentiels postsynaptiques excitateurs de champ dans la région CA1 évoqués par une stimulation collatérale de Schaffer. La PLTa été renforcé en réponse à l'infection maternelle. Ces résultats sont cohérents avec les données d'expression génique de l'hippocampe issues de l'analyse de séquençage d'ARN, qui indiquent un développement accéléré des synapses de glutamate matures à PD 23 chez les mâles et les femelles issus de mères infectées. Ces résultats expliquent, au moins en partie, l'amélioration de la mémoire spatiale chez les petits juvéniles de mères infectées par *H. bakeri*.

En réponse à une infection par *H. bakeri*, les mères infectées déclenchent une forte réponse immunitaire Th2/Treg. Comme le système immunitaire maternel influence le système immunitaire de la progéniture qui, à son tour, peut fortement influencer le développement neurologique et le comportement, l'impact de l'infection maternelle sur la capacité des jeunes

mâles et femelles à résister à l'infection directe a été évalué en tant qu'indice de l'immunité périphérique, et l'environnement neuro-immunitaire de l'hippocampe des petits a également été exploré. À PD 27, la progéniture a été directement infectée par *H. bakeri*. Un mois plus tard, la progéniture des mères infectées était plus résistante à l'infection, comme en témoigne la diminution du nombre de vers et de la fécondité, ce qui indique une protection fonctionnelle contre l'infection directe par *H. bakeri*, en accord avec le transfert maternel d'IgG1 spécifiques à *H. bakeri*. À PD 22, l'environnement neuro-immunitaire a également été modifié par l'infection maternelle. L'immunohistochimie a révélé un plus grand nombre de microglies et d'astrocytes, ainsi qu'un plus grand pourcentage de microglies exprimant le marqueur CD206 qui est généralement régulé à la hausse en réponse aux cytokines Th2. En outre, les données d'expression génique de l'hippocampe ont montré une régulation accrue de la voie de signalisation TGF-β associée à l'immunorégulation. Ces derniers résultats indiquent que l'environnement neuro-immunitaire des jeunes non infectés imite l'environnement immunitaire périphérique de leur mère infectée.

La dernière étude a permis de déterminer si l'amélioration de la mémoire spatiale des jeunes mâles et femelles issus de mères infectées par *H. bakeri* était toujours évidente à l'âge adulte. Le test du labyrinthe de Barnes a révélé qu'en réponse à l'infection maternelle, les femelles non infectées âgées de trois mois avaient une meilleure mémoire spatiale, mais pas les mâles. Cette différence entre les sexes peut s'expliquer en partie par l'influence des hormones sexuelles sur le système immunitaire.

Ensemble, ces résultats originaux démontrent qu'une infection maternelle par un nématode gastro-intestinal pendant la grossesse et l'allaitement renforce la mémoire spatiale chez les descendants non infectés. Les données suggèrent une voie causale potentielle pour expliquer ce phénomène, selon laquelle la réponse immunitaire Th2/Treg de la mère infectée est imitée dans le cerveau du petit non infecté, entraînant une PLT hippocampique améliorée qui, à son tour, favorise la mémoire spatiale. Ces résultats mettent en lumière un avantage possiblement non apprécié de l'infection maternelle par des nématodes gastro-intestinaux et soulignent une augmentation potentielle de la condition physique de la progéniture.

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Contributions of Authors

The thesis is written by Sophia C. Noel in the manuscript-based style as per the guidelines of McGill University. It contains three manuscripts, all of which I am the first author, and my academic supervisor, Dr. Marilyn E. Scott, is the senior author.

My first manuscript (Chapter III) is co-authored with Dr. Scott's past PhD student, Dr. Manjurul Haque, and honour's student, Liana Fortin-Hamel. For this study, I conceived and designed the study, identified, ordered, and set up behavioural equipment and tracking software from Noldus, conducted experimental work (including ordering mice, maintaining the mouse colony [i.e. cage cleaning, weaning, euthanasia], culturing the parasite, infecting mice via oral gavage, confirming successful infection [eggs per gram of feces and worm burden count], recording mass/crown-rump length of mice, and performing behavioural experiments), analyzed the data, interpreted the results, and drafted the manuscript. L.F.H. assisted with infecting mice on occasion, assisted with behavioural experiments, and after receiving training and guidance from me, extracted data from the behavior videos using the Ethovision XT tracking software. M.H. provided me with training for mouse colony maintenance, infecting mice and culturing the parasite, and provided input on the study design, and suggestions that have been incorporated into the publication. M.E.S. provided input on the study design and data interpretation, provided critical suggestions that have been incorporated into the manuscript, and obtained funding for the research.

My second manuscript (Chapter IV) is co-authored with Dr. Jessica Ewald, a bioinformatician at McGill University's Institute of Parasitology, and my collaborators at the Montreal Neurological Institute, Dr. Timothy E. Kennedy and Dr. Austen J. Milnerwood, as well as Dr. Kennedy's post-doc, Dr. Jean-David M. Gothié, and PhD student, Jeanne F. Madranges. For this study, I conceived and designed the study, conducted experimental work, analyzed the data, interpreted the results, and drafted the manuscript. I conducted all the work with the live mice, including ordering mice, maintaining the mouse colony, infecting mice, confirming infection, recording mass/crown-rump length of mice, and transporting mice to the MNI. For the gene expression study, I collected pup hippocampal samples and submitted them to Genome Quebec for RNA extraction and sequencing. J.E. performed the raw data processing of the

FASTQ files, and after providing me with these files, I analyzed the data. J.E. also provided helpful suggestions for the manuscript. For the LTP study, after transporting the pups to the MNI's animal facility, J.F.M. performed the electrophysiology experiment with my assistance. J.F.M. also taught me how to extract the raw data and assisted with data extraction. I analyzed the data and interpreted the results. For the resistance study, I conducted the entire study myself, which included infecting pups, assessing parasite infection intensity (eggs per gram of feces and worm burden count), and performing ELISAs. For the neuroimmune study, J.M.G. provided input on study design and initial training for the immunohistochemistry (IHC) experiment (intraperitoneal injection, transcardiac perfusion, tissue preparation, IHC, and image capture and analysis), but did not assist me with the experiment. M.E.S, T.E.K. and A.J.M. provided input on study design, data interpretation and feedback and suggestions for the manuscript. M.E.S and T.E.K. obtained funding for the research.

My third manuscript (Chapter V) is co-authored with Dr. Scott's past honour's student, Ryan LaFrancois. For this study, I used littermates from my second study (Chapter IV). I conceived and designed this study, conducted experimental work, analyzed the data, interpreted the results, and drafted the manuscript. For this study, I trained R.L. for the Barnes Maze Test protocol and data extraction from the behavior videos using the Ethovision XT software and he assisted me with these. M.E.S. provided input on the study design, data interpretation, and critical suggestions that have been incorporated into the manuscript, and obtained funding for the research.

Statement of Originality

Using an *in vivo* model where pregnant and lactating mice were infected with the gastrointestinal (GI) nematode, *Heligmosomoides bakeri,* that remains in the maternal intestine and does not directly infect the offspring, I have made the following novel contributions to science.

1. This is the first study to explore the impact of maternal GI nematode infection on uninfected offspring spatial memory, hippocampal long-term potentiation, and neuroimmunity.

2. The ability of rodents to retain object location memories for at least two hours has previously been shown to first occur at postnatal day (PD) 24[1]. However, I found that, in response to maternal *H. bakeri* infection, both juvenile male and female offspring at PD 17 retained object location memories for three hours in the Object Location Test but, as expected, offspring of uninfected mothers did not. This demonstrates enhanced spatial memory and suggests that the maturational process needed to recall object location memories for three hours occurred earlier as a result of this maternal infection.

3. In response to this maternal infection, I observed that both juvenile male and female offspring at PD 34 exhibited enhanced long-term spatial reference memory, as they retained their ability to find an escape location in the Barnes Maze Test (BMT) for one week but offspring from uninfected mothers did not. As retention of spatial reference memories for one week does not normally occur in rodents until PD60[2], my results suggest accelerated development of spatial memory in these juvenile offspring in response to maternal infection. These novel findings shed light on a possible unappreciated benefit of maternal GI nematode infection on offspring neurodevelopment and cognition, however, the mechanism was unknown.

4. A previous study observed that maternal *H. bakeri* infection up-regulated genes associated with long-term potentiation (LTP) in the whole brain of seven-day old neonates[3]. Given that hippocampal LTP of glutamatergic synapses is a form of activity-dependent synaptic plasticity that is positively associated with spatial memory[4], I used

acute hippocampal slices from PD 21-24 male pups to record field excitatory postsynaptic potentials in the CA1 region evoked by Schaffer collateral stimulation. I found that maintenance of LTP for >60 mins was only observed in 14% of pups from uninfected mothers compared with 71% of pups from *H. bakeri* infected mothers. As the capacity to maintain LTP for >60 mins in mice normally occurs at 4-5 weeks of age[5], my data indicate that maternal infection accelerated the capacity to induce and maintain LTP.

5. Findings of enhanced LTP from the electrophysiology experiment were consistent with my hippocampal RNA-seq data from PD 23 male and female pups which indicated accelerated development of glutamatergic synapses in offspring of infected mothers, relative to those from uninfected mothers. These novel findings explain, at least in part, the enhanced spatial memory I previously observed.

6. As the maternal immune system influences the offspring immune system that, in turn, can strongly influence neurodevelopment and behaviour[6], I assessed the impact of maternal infection on the ability of juvenile male and female offspring to resist direct infection as an index of peripheral immunity. A previous study observed that parasite-specific IgG1 is transferred (via nursing) from the *H. bakeri* infected dam to pre-weaned 10-day old neonates, protecting them against *H. bakeri*[7]. To determine if this resistance was maintained in weaned, juvenile offspring in my model, I infected juvenile male and female offspring of infected and uninfected mothers with *H. bakeri* at PD 27 and necropsied them one month later. I observed that the offspring of the infected mothers were more resistant to infection as evidenced by lower worm burdens and fecundity, indicating functional protection against direct *H. bakeri* infection. This was consistent with the maternal transfer of *H. bakeri*-specific IgG1, which I detected in the serum of PD 24 uninfected offspring of infected mothers, indicating transfer of protective antibodies from the *H. bakeri* infected mother to their offspring.

7. *H. bakeri* infection results in a strong Th2/Treg immune response in the infected mother[8,9] and a previous study observed that maternal *H. bakeri* infection up-regulated genes associated with Th2/Treg immunity in the whole brain of seven-day old

neonates[3,10], indicating the neuroimmune system may be influenced. To assess whether maternal *H. bakeri* infection influenced the hippocampal neuroimmune system of uninfected male and female offspring, I assessed glial cell density at PD 22. Glial cells (microglia, astrocytes and oligodendrocytes) are key cellular components of the neuroimmune system that also have vital roles during neurodevelopment. Thus, alterations to their differentiation and/or function in response to immune stimuli from the infected mother could alter the developmental trajectory of neural circuits and associated behavioral outcomes. In response to maternal *H. bakeri* infection, immunohistochemistry (IHC) revealed greater numbers of microglia and astrocytes, and a greater percentage of microglia expressing the CD206 marker that is typically increased in response to the Th2 cytokine, IL-4[11-13]. Further, hippocampal RNA-seq data from PD 23 male and female pups indicated up-regulation of the TGF-β signaling pathway, which is associated with immunoregulation. These findings suggest the neuroimmune environment in the hippocampus of juvenile uninfected pups mimic the peripheral immune response of the infected mother.

8. Further, TGF-β signaling is also critical for differentiation, development and function of neurons and glia[14,15]. Thus, the up-regulation of this pathway may in part explain the up-regulation of genes associated with neurogenesis, gliogenesis and myelination that I also observed. As neurogenesis is positively associated with spatial memory performance[16], and early development of hippocampal myelination promotes excitatory synaptic transmission and cognitive function[17], this may contribute to the enhanced LTP and spatial memory in these offspring.

9. Finally, I assessed whether the enhanced spatial memory of juvenile male and female offspring of *H. bakeri* infected dams was still evident at adulthood. The BMT revealed that female adult offspring of *H. bakeri* infected dams retained enhanced spatial reference memory and also exhibited signs of reduced anxiety-like behaviour compared to females of uninfected dams. I observed no differences in the behaviour of adult male offspring of infected vs. uninfected dams. I hypothesize that sex hormones may at least in part explain the sex-specific differences in behavioural responses to maternal infection of adult offspring.

10. With respect to the uninfected adult offspring, I also observed that they had lower mass and crown-rump length in response to maternal *H. bakeri* infection, an observation that has previously been found in fetal and juvenile uninfected offspring[18-20], but not in adult offspring.

11. Taken together, my novel findings highlight that a maternal GI nematode infection enhances spatial memory of uninfected offspring, possibly through promotion of hippocampal LTP and upregulation of genetic markers associated with neurogenesis, gliogenesis and myelination. As an *H. bakeri* infection is associated with high levels of TGF- β and IL-4[21], and these cytokines are known to have measurable downstream effects on LTP and spatial memory[22-27], our evidence indicates this maternal infection may be influencing the cognitive function of offspring possibly through transfer of a Th2/Treg immune phenotype that protects the offspring from direct infection and extends to their developing brain.

12. My study provides evidence that maternal GI nematode infection during pregnancy and lactation may provide benefits to neurodevelopment and cognitive function of offspring, countering the assumption that maternal infections only have harmful effects on offspring. Given the immunoregulatory nature of this parasite, which extends to the offspring brain, my findings may be valuable in efforts to prevent the development of neurological disorders associated with immune dysregulation, such as multiple sclerosis and autism spectrum disorder.

NOTE: References for Statement of Originality can be found in the "Master List of References for All Non-Manuscript Sections" located at the end of the document.

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List of Abbreviations

aCSF	Artificial cerebrospinal fluid
AMPA	3-hydrozy-5-methylisoxazole-4-propionic acid
ASD	Autism spectrum disorder
BDNF	Brain derived neurotrophic factor
BMT	Barnes maze test
BSA	Bovine serum albumin
CA	Cornu Ammonis
DEG	Differentially expressed gene
CNS	Central Nervous System
DEA	Differential expression analysis
DG	Dentate gyrus
EAE	Encephalomyelitis
EC	Entorhinal cortex
ED	Embryonic day
ELC	Early Learning Composite score
ELISA	Enzyme-linked immunosorbent assay
EPG	Eggs per gram of faeces
fEPSPs	field excitatory postsynaptic potentials
FoxP3	Transcription factor forkhead box P3
FRQNT	Fonds de Recherche du Québec Nature et Technologies
GABA	Gamma-aminobutyric acid
GD	Gestation day

GFAP	Glial fibrillary acidic protein
GI	Gastrointestinal
GLMM	General linear mixed model
GO BP	Gene ontology biological process
HES	*H. bakeri* excretory-secretory antigen
HFS	High frequency stimulation
HIHS	Heat-induced horse serum
Iba1	Ionized calcium-binding adaptor molecule 1
IBD	Irritable bowel disease
IFN-γ	Interferon gamma
IGF1	Insulin-like growth factor 1
IHC	Immunohistochemistry
IL	Interleukin
IL-1β	Interleukin 1 beta
ILC2s	type 2 innate lymphoid cells
I/O	Input/output
KEGG	Kyoto encyclopedia of genes and genomes
LM	Linear model
LMM	Linear mixed model
logCPM	Log2-counts-per-million
LPS	Lipopolysaccharide
LTD	Long-term depression
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase

MIA	Maternal immune activation
miRNA	Micro ribonucleic acid
MMc	Maternal microchimeric cells
MNI	Montreal Neurological Institute
MS	Multiple sclerosis
NB	Negative binomial
NMDA	N-methyl-D-aspartic acid
NSERC	Natural sciences and engineering research council of Canada
OCT	Optimal cutting temperature
OFT	Open field test
OLT	Object location test
PBS	Phosphate-buffered saline
PD	Post-natal day
PFA	Paraformaldehyde
PI	Post-infection
Poly IC	Polyinosinic:polycytidylic acid
PPF	Paired-pulse facilitation ratio
RNA	Ribonucleic acid
RT	Room temperature
TGF-β	Transforming growth factor-β
Th	T helper
TMB	3,3′,5,5′-Tetramethylbenzidine
TNF-α	Tumor necrosis factor alpha
Treg	Regulatory T cell

Chapter I - Introduction

Gastrointestinal (GI) nematodes are ubiquitous in vertebrates and developing countries, and globally, it is estimated that at least 1.5 billion people (24% of the world's population) are infected[28,29]. Mortality attributable to intestinal nematodes is relatively low compared to other prevalent infectious diseases in developing countries[30] [31]. However, GI nematodes are associated with important co-morbidities, particularly in individuals who are malnourished and have heavy worm burdens[28,29]. Pregnant women are at high risk of heavy infections and nutrient deficiencies[32,33], and it is estimated that at least one-third of all pregnant women in endemic settings are infected with hookworm[34]. Despite this, our understanding on the significance of GI nematode exposure for the overall health of the mother and child is limited[35,36], and in particular, the influence on offspring neurodevelopment and cognitive function has been understudied in the field[37,38], and has never been studied in an experimental setting.

Brain development is a highly plastic process that starts *in utero* and continues postnatally. Events that occur during this period can modulate the functional maturation of the brain and determine its lifelong integrity. In this context, epidemiological reports suggest an association between neurodevelopmental disorders, like autism spectrum disorder (ASD) and schizophrenia, and prenatal exposure to viral or bacterial pathogens [39-41]. Although the link between maternal infection and neurodevelopmental disorders is not fully understood, rodent models have highlighted that it is the maternal immune response, not a specific pathogen, which is a risk factor for the development of these disorders[42]. In response to maternal viral and bacterial pathogens, a strong T helper type 1 (Th1) and Th17 pro-inflammatory immune response is observed in the mother which is mimicked peripherally and in the developing brain of the offspring, and ultimately leads to irreversible neurodevelopmental defects and the emergence of behavioral abnormalities and cognitive impairments[43-48]. Of note, the hippocampus, the brain region associated with spatial navigation, learning and memory[49], is a plastic and vulnerable structure that gets damaged by a variety of stimuli[50], including inflammatory cytokines[6,51-53]. Exposure of pregnant dams to viral and bacterial infections increased pro-inflammatory cytokines in the offspring hippocampus, most notably interleukin (IL)-1β, IL-6, and tumor necrosis factor alpha (TNFα), and activated microglia and astrocytes, and this

neuroinflammatory response was associated with impaired hippocampal long-term potentiation (LTP) and spatial learning and memory deficits [45,54-60]. Interestingly, administration of anti-inflammatory drugs or cytokines to the mother attenuated the neuroinflammatory response in the offspring hippocampus and rescued the LTP and cognitive deficits[56,58,60].

While infections that induce pro-inflammatory responses in both the mother and offspring are detrimental to offspring neurodevelopment and cognitive function[44,47], studies focused on pathogens that induce immune tolerance in their host, such as GI nematodes, are lacking. Effective anti-nematode immunity typically relies on a strong type 2 immune response, characterized by Th2 lymphocytes which secrete IL-4, IL-5, IL-9, IL-10, and IL-13 cytokines and high antibody titers[61]. However, GI nematodes can suppress this type 2 immune pathway by inducing an immunoregulatory network, associated with regulatory T cells (Tregs) and high levels of the potent immunoregulatory cytokine, transforming growth factor-β (TGF-β), which ensures their survival [62,63]. Of note, while increased levels of the pro-inflammatory cytokine, IL-1β, impairs hippocampal LTP and spatial memory[6,51-53], increased levels of the anti-inflammatory cytokine, IL-4, enhances hippocampal LTP[24] and spatial memory[22,25], and IL-13 and TGF-β also regulate cognitive processes, including LTP and memory[23,27]. This raises the possibility that a maternal GI nematode infection, which induces an immune tolerant environment in the pregnant mother [8], may benefit, not harm, offspring neurodevelopment. Previous findings support this hypothesis.

Using the murine laboratory model, *Heligmosomoides bakeri* (also known as *Heligmosomoides polygyrus*), a strictly intestinal nematode with a direct lifecycle[64], brain gene expression data from one-week old male pups indicated an up-regulation of five key interacting pathways associated with LTP in response to maternal infection[3]. Considering LTP of hippocampal glutamatergic synapses is a form of activity-dependent synaptic plasticity that is positively associated with spatial learning and memory[4,65], this raises the possibility that this maternal infection may be beneficial for offspring cognition. It was also reported that maternal infection with *H. bakeri* results in the transfer (via nursing) of maternally derived parasite-specific antibodies[26], which protects 10-day old neonates from *H. bakeri* infection. It is possible that immune molecules from the *H. bakeri* infected mother may also reach the brain of uninfected neonates, as brain gene expression data from one-week old male pups indicated that Th2/Treg pathways, and genes for IL-4, TGF-β and Foxp3 (a biomarker expressed by Tregs)

were up-regulated in response to a maternal *H. bakeri* infection[3,10]. In contrast, the pro-inflammatory cytokine, IL-1β, as well as Th1/Th17 pathways associated with inflammation were down-regulated[3,10]. Together these observations indicated that the Th2/Treg immune response observed in the *H. bakeri* infected mother[8,9] may be mimicked in the brain of their neonates, and this altered neuroimmune environment may be beneficial for neurodevelopment and cognition. If this is found to be true, it indicates that a maternal GI nematode infection might have evolutionary advantages for the offspring. Further, if this maternal GI nematode infection can regulate the neuroimmune environment of offspring, this could prove valuable in efforts to prevent the development of inflammation-associated neurological disorders like ASD and multiple sclerosis.

Rationale and Research Objectives

Maternal infections influence the developing immune system that, in turn, can strongly influence neurodevelopment and cognitive function[6,43]. Given that immune molecules are transferred from the GI nematode infected mother to their neonate, protecting them from direct infection[26], given that brain gene expression data from neonates suggests that a maternal GI nematode infection induces a Th2/Treg biased neuroimmune environment that may promote LTP[3], and given that hippocampal LTP is positively associated with spatial memory[4,65] and enhanced by Th2/Treg cytokines[22-25,27], my thesis explored the following objectives:

Study 1 objective: To determine if maternal *H. bakeri* infection influences spatial learning and memory of uninfected male and female juvenile offspring.

Study 2 objectives: To determine if maternal *H. bakeri* infection influences:

a) Hippocampal LTP of uninfected male juvenile offspring.
b) Hippocampal neuroimmune environment of uninfected male and female juvenile offspring.
c) Resistance to *H. bakeri* infection of male and female juvenile offspring as an index of peripheral immunity.

Study 3 objective: To determine if maternal *H. bakeri* infection influences spatial learning and memory of uninfected male and female adult offspring.

NOTE: References for Chapter I - Introduction can be found in the "Master List of References for All Non-Manuscript Sections" located at the end of the document

Chapter II - Literature Review

2.1. Gastrointestinal nematodes

2.1.1. Prevalence and immunity

Gastrointestinal nematodes (intestinal worms) are considered neglected tropical diseases and cause extensive morbidity in both humans and livestock[28]. Infections are most common in impoverished rural areas of Sub-Saharan Africa, Latin America, Southeast Asia, and China[66]. This is associated with the poor housing, overcrowded living conditions, lack of adequate sanitation and hygiene, and poor education and health care in these areas, as well as the warm and humid climatic conditions[29]. Infection often occurs through ingestion or skin contact of parasite eggs or larvae that typically live in warm, moist soil. After various migratory/molting events by the larvae, they return to the intestines where they mature into adult worms. Adult worms are often capable of long-lived survival in their hosts where they feed and produce eggs which are passed in the host feces and contaminate the soil to continue the cycle [28].

Currently, these infections result in major economic losses in the livestock industry [67,68] and due to prophylactic mass treatment of livestock with the same group of anthelmintics, drug resistance is a widespread issue [69,70]. In humans, globally more than 1.5 billion people (24% of the world's population) are infected with GI nematodes, namely *Ascaris lumbricoides*, *Trichuris trichiura*, and hookworms (*Necator americanus* and *Ancylostoma duodenale*)[28,29]. While the intensity of *A. lumbricoides* and *T. trichiura* decrease in adulthood [71], hookworm tends to increase with age [35,72], and it is estimated that at least one-third of all pregnant women in endemic settings are infected with hookworm[34]. Worm burdens exhibit a highly aggregated distribution[73-75] whereby most people harbor relatively few worms in their intestines, and are usually asymptomatic, whereas a small portion of the population harbor disproportionately large worm burdens, and these individuals can present with a range of symptoms including diarrhoea, abdominal pain, malnutrition, and impaired growth and development [28]. Importantly, individuals suffering from nutritional deficiencies and anemia have increased susceptibility to infection, which promotes the further loss of nutrients, leading to reduced growth and poor nutritional status as part of a vicious cycle[29,76].

Despite GI nematode infections being associated with important co-morbidities, in comparison to other prevalent infectious diseases in developing countries, such as Tuberculosis (∼1 in 10 individuals) and Malaria (∼1 in 100), GI nematode-induced mortality is relatively rare (<1 per 20,000) [30,77]. This also holds true for heavily infected individuals, as the Global Burden of Disease study in 2001 observed that, despite 58.1 million, 26.6 million and 59.9 million people suffering from high intensity *A. lumbricoides*, *T. trichiura* and hookworm infections, respectively, only 3000 deaths were attributable to each species[31]. This low mortality rate is surprising when considering the host must accommodate a relatively large pathogen, which is capable of establishing a chronic infection that can last up to several years in the host[31].

Effective anti-nematode immunity typically relies on a strong type 2 immune response, characterized by Th2 lymphocytes which secrete IL-4, IL-5, IL-9, IL-10, and IL-13 cytokines and high antibody titers[61]. However, GI nematodes can suppress this type 2 immune pathway by inducing an immunoregulatory network, which ensures their survival [62,63]. Due to the immunoregulatory effects of GI nematodes, they can suppress immunity against co-infections with bacteria, viral and protozoan pathogens, which may lead to an insufficient control of these co-infections and increased pathology[78]. Further, vaccine efficacy can also be compromised by nematode infections due to suppressed immune responses[78]. Interestingly, despite important co-morbidities associated with GI nematodes, that are not to be ignored, diseases associated with chronic inflammation/hyperimmunity, such as allergies, autoimmune diseases, and inflammatory bowel diseases (IBD) are all reduced in prevalence in areas where GI nematodes are endemic[79,80]. Further, animal models and pre-clinical trials have suggested a beneficial effect of GI nematode infections on autoimmune diseases, allergies and IBD [81]. As such, GI nematode therapy has been suggested as a possible treatment method for these chronic inflammatory/ hyperimmune-associated disorders in humans, and efforts are currently underway[81-86].

Additional to the potential importance of GI nematodes for treating hyperimmune-associated disorders, it is strongly believed that due to their immunoregulatory influence, these organisms may also have an unexpectedly broad impact on many areas of human and animal biology[79,80]. It has been proposed that the perinatal period is of particular importance, as maternal exposure to GI nematode infections can have important long-term effects on the development of the immune system[35,87,88], which may in turn influence brain development and cognitive function[6]. Given the interest in GI nematode therapy for ameliorating chronic

inflammatory/ hyperimmune-associated disorders [80,81], and given that a large number of women in endemic settings are infected with GI nematodes[34], it is of great interest to understand the influence that a maternal GI nematode infection has on cognitive function of their offspring.

2.1.2. Pregnancy, GI nematode infection and offspring outcomes

Pregnancy is associated with shifts in immune responses. During the luteal phase of the menstrual cycle, regulatory and Th2 cell responses increase[89,90]. If conception occurs, these shifts continue through pregnancy and help to suppress Th1 responses, increasing maternal tolerance of an immunologically distinct fetus[89,90]. Similar to the developing fetus, GI nematodes are also immunologically foreign to their host, and are associated with a general shift from Th1 toward Th2 responses and an increase in the suppressive activity of regulatory T cells, to modulate both Th1 and Th2 responses[61-63,90]. Interestingly, pregnancy is associated with a heavier worm burden in mice [18], and in humans, it was observed that pregnancy increases risk of GI nematode infection in Sub-Sahara African women[91]. Further, a longitudinal study among Bolivian forager-horticulturalist women observed that the GI nematode, *A. lumbricoides*, favoured conception, implantation and overall fecundity among women[90].

Despite the possible benefits of GI nematodes for fertility, likely associated with immunomodulation[90], GI nematode infection during pregnancy can be associated with nutritional deficiencies which increase the risk of delivering premature or low birth weight infants [92], who themselves are at increased risk of poor growth and development [73]. Thus, the World Health Organization recommends anthelmintics for pregnant women after their first trimester [93]; however, the benefits remain unclear [35,94,95]. Although some studies have attempted to understand the influence that maternal GI nematode infection has on offspring health and development, findings are inconclusive and sometimes contrasting[35,95-100], and the influence on brain development and cognitive function has been understudied.

It is understood that GI nematode infections leave a long-lasting immunological footprint on their hosts[87]. Maternal transfer of immunity both *in utero* and via nursing provides critical sources of early life immune education and although understudied, evidence from experimental mouse models indicates that maternal GI nematode infections can result in an altered immune profile in their offspring which can potentially shape how they respond to conditions throughout

life[87]. For example, maternal infection with the murine GI nematode, *H. bakeri,* provides high levels of nursing-acquired antibody-mediated protection to offspring against this infection[7]. Similarly, maternal infection with the GI nematode, *Nippostrongylus brasiliensis*, resulted in the transfer (via nursing) of maternally derived Th2 lymphocytes[101,102], which provided long-lasting cellular immunity against this nematode[101]. In addition to the passive transfer of antibodies and maternal cells, maternal GI nematode infection can also shape offspring immunity via the microbiome[87]. GI nematodes coexist with trillions of microbes in the GI tract and their impact on the diversity and abundance of the microbiome has been shown for several nematode parasites across a diverse range of hosts[103]. As a mother's microbiome is the initial source of the neonatal microbiome[104], it is not surprising that maternal infection with *N. brasiliensi*[102] or *H. bakeri*[9,105] altered the microbiome of both the infected mother and their offspring. Of further interest, rodent models indicate that maternal helminth infections can protect against inflammatory responses in offspring, as maternal infection with the cestode, *Hymenolepsis diminuta*[106], and the trematode, *Schistosoma mansoni*[107], protected offspring from harmful neuroinflammatory responses to early-life infection and respiratory allergies, respectively. A similar effect was also seen in humans, whereby anthelmintic treatment of Ugandan mothers increased the prevalence of infantile eczema and respiratory wheeze compared with that seen in a placebo-controlled group[95,108]. Considering the important relationships among the gut microbiome, immune system and brain during (and beyond) development[109-111], and knowing that maternal GI nematode infections can influence both the immune system and gut microbiome of their offspring[112,113], it is of great interest to determine whether changes in neurodevelopment and cognitive function of uninfected offspring are also evident.

A small number of studies have directly infected adult rodents with GI helminths, including *H. diminuta, Ancylostoma ceylanicum, N. brasiliensis and Strongyloides ratti,* and observed varying results, from enhanced[114], to impaired[115,116], to no differences[117] in cognitive function respectively. However, in addition to using different helminths, different experimental approaches were taken, including time between infection and behavioural testing. For example, the studies which found an impairment of helminth infection on spatial memory recorded observations within 1-14 days post infection[115,116], whereas the study that found an enhancement in spatial memory, recorded observations 15 months post infection[114]. It is possible that if behavioural tests are performed shortly after infection - at a time when the

helminth is invading host tissue and maturing and the host is utilizing resources to defend against the helminth[30,118] - there may be a negative influence on cognition. Conversely, if behaviour tests are performed months after infection, once the host and mature helminth are co-existing and the helminth is having an immunoregulatory effect, cognitive benefits may be observed. Despite the need for further exploration in these direct infection models, understanding the influence that a maternal GI nematode infection may have on offspring cognitive function is of great importance, and to the best of my knowledge, no controlled experimental studies have assessed this.

With regard to studies in humans, while there is some evidence from school-age children that direct infection with intestinal nematodes impairs cognition [119,120], meta-analyses of the human studies conclude that the body of evidence does not support this claim [96,97]. With respect to the influence of a maternal GI nematode infection on infant cognition, there are two epidemiological studies of relevance that were both performed in Africa[37,38]. Of note, these two studies had differing testing procedures for how they scored infant cognitive function. Mireku et al.[37] combined scores for visual reception, fine motor, receptive language and expressive language scales to form the Early Learning Composite (ELC) score, which was indicative of early cognitive function. They assessed the influence of maternal hookworms on ELC[37]. Alternatively, Nampijja et al. [38] did not combine scores of different tasks, and assessed fine motor, gross motor, language, self-control, self-care, self-recognition and object permanence separately. They analyzed the influence of maternal hookworm, *A. lumbricoides*, *T. trichiura*, and *Strongyloides stercoralis* on infant scores in these cognitive tasks[38]. Mireku et al. [37] found a negative association between maternal hookworm infection and infant ELC scores, however, Nampijja et al.[38] did not find an association between maternal hookworm infection on infant scores in any of the cognitive tasks. The only correlation Nampijja et al.[38] observed was a negative association between maternal *S. stercoralis* infection and infants' language. One explanation behind the contrasting observation associated with the influence of maternal hookworm infection on infant cognition in these two studies, may be that Nampijja et al. [38] excluded pregnant women presenting with moderate to severe anemia. They also included some maternal and infant characteristics (such as maternal hemoglobin level and birth weight) in their final statistical model. Maternal nutritional deficiencies, particularly iron deficiency, is important to control for as it is known to have negative impacts on offspring cognition [121-127]. It can thus

be speculated that if Mireku et al. [37] controlled for maternal anemia, they may not have found an association between maternal hookworm infection and infant cognition. These differing results emphasize the need for controlled experiments to better understand the relationship between maternal GI nematode infection and offspring cognitive function.

2.2 *Heligmosomoides bakeri*; a model organism

As most GI nematodes have co-evolved with, and are closely adapted to their human host, they are difficult to study in the laboratory[64]. However, model organisms such as *H. bakeri*, a strictly local intestinal parasite of wild mice, provides an invaluable system to explore the mechanisms of immunity and immune evasion in nematode infections [21,64]. *H. bakeri* (also known as *Heligmosomoides polygyrus* and previously known as *Nematospiroides dubius*) is phylogenetically placed in the same Suborder, Trichostrongylina, as the ruminant GI nematodes *Haemonchus contortus* and *Teladorsagia circumcincta* and within the same Order, Strongylida, as the human hookworm parasites [64,128]. *H. bakeri* is an appropriate model of these chronic helminthiases as primary infections can persist for many months in susceptible strains of mice [64]. Due to its immunoregulatory ability, *H. bakeri* has been shown to prevent or treat a number of hyperimmune-associated disorders, including asthma, allergies, type 1 diabetes, multiple sclerosis (MS) and IBD in mouse models [81,129-133].

2.2.1. *Heligmosomoides bakeri* life cycle

In a lab setting, *H. bakeri* is introduced by oral gavage with infective third stage larvae (L_3) [21,64]. Within 24 hours following ingestion, larvae penetrate the submucosa of the small intestine and reside under the muscularis externa layer where they undergo two developmental molts before returning to the gut lumen as adult worms at 6-8 days post-infection (PI) [134]. The adult worms do not migrate out of the intestinal lumen; they secure themselves by coiling around the intestinal villi and feed on the epithelial cell layer of the small intestine (not on host ingesta or blood)[135]. Between days 9-11 PI, mature adult male and female worms mate in the anterior duodenum and produce eggs, which are passed in the faeces. Eggs hatch in the environment within 24-36 hours and undergo two molts to become infective L_3 within 6-7 days, thus

continuing the lifecycle [64,136]. As larvae must develop in the environment before becoming infectious, these parasites cannot be transmitted directly nor vertically.

2.2.2. Host immune response to *Heligmosomoides bakeri*

Similar to most GI nematodes, immunity against *H. bakeri* relies on a strong type 2 immune response and is CD4+ Th2 cell mediated, however, its effectiveness varies based on the frequency of infection [21,64]. Primary *H. bakeri* infection stimulates a highly polarized Th2 immune response which is characterized by type 2 innate lymphoid cells (ILC2s), elevated IL-4, IL-5, IL-9, IL-10 and IL-13 cytokine secretion, and eosinophilia and mastocytosis in intestinal tissue [64,137,138]. However, a primary infection is generally non-resolving and becomes chronic [137] because adult *H. bakeri* release immunosuppressive factors that minimize the protective Th2 response [139]. Immunity is often observed in a challenge infection where mice are re-infected after elimination of adult worms by an anthelmintic drug, or in a trickle infection, where mice are repeatedly infected over time [21,140]. After challenge or trickle infection the parasite is controlled by a rapid Th2 memory response involving IL-4 secretion by memory CD4+ Th2 cells [137] together with secretion of *H. bakeri*-specific IgG1 and IgE antibodies by plasma cells [141-143]. This memory response results in the rapid development of granulomas around invading larvae which contain neutrophils, dendritic cells, eosinophils, alternatively activated macrophages, IgG1, and CD4+ Th2 cells [62,64,144]. Any larvae reaching maturity are exposed to Th2 effectors, enhanced mucous secretion from goblet cells, and increased intestinal smooth muscle contraction that results in expulsion of worms from the intestine [144,145]. Trickle infections are ideal when trying to simulate natural transmission in laboratory settings, as wild mice are frequently exposed to *H. bakeri* infection throughout their lives[140]. During trickle infections, animals are repeatedly infected over time and therefore harbor both larval and adult stages simultaneously, and as such, the host immune system is continuously stimulated by the larvae, while adult worms are simultaneously inducing immunomodulatory effects[140].

IL-4 has been identified as the most vital cytokine for protection against *H. bakeri* infection[64], as *in vivo* treatment of mice with anti-IL-4 and anti-IL-4 receptor antibody, which blocks IL-4 activity, results in loss of protection against a secondary infection[146]. With respect to antibodies, high serum levels of IgE and IgG1 antibodies are observed, with primary *H. bakeri*

infection eliciting an extraordinary increase in nonspecific serum IgG1 levels[143]. Using selective isotype knockout mice given a secondary *H. bakeri* infection, it was found that IgE had no role in protection, and IgA had a minor role, leaving IgG as the major class-switched isotype leading to protection [143]. Interestingly, transfer of serum from mice infected with a single *H. bakeri* infection, that contains high levels of nonspecific IgG1 does not protect naive animals from infection[143,147]. In contrast, serum raised after multiple *H. bakeri* infections is protective against adult worm survival when transferred into naive recipients[7,147], as the higher ratio of parasite-specific to nonspecific IgG1 following repeated infection is vital in protection against this parasite[64,148].

2.2.3. Immune regulation by *Heligmosomoides bakeri*

During a primary infection, by the time the Th2 immune response is in full effect, the adult worms have induced an immunoregulatory network involving proliferation of Foxp3+ CD4+ regulatory T (Tregs) cells, tolerogenic dendritic cells, and the potent immunoregulatory cytokines IL-10 and TGF-β [21,64]. This results in the downregulation of effector CD4+ Th2 cells leading to suppression of host immunity, which allows for parasite tolerance and survival [149,150]. *H. bakeri* is also capable of directly regulating the immune response through release of excretory secretory products, including a TGF-β mimic, which further aids parasite survival [137,151]. TGF-β is particularly important for parasite survival in its host as inhibition of TGF-β signaling during *H. bakeri* infection reduces adult worm burden and results in an increased Th2 response[152]. Further evidence of the importance of TGF-β in infection-mediated immunomodulation is observed in colitis models, as even in the absence of IL-10, *H. bakeri* is able to minimize experimentally induced colitis [153]. However, *H. bakeri* infection fails to improve colitis in TGF-β receptor knockdown mice[151].

2.2.4. *Heligmosomoides bakeri* and the gut microbiome

It has been shown that GI nematodes modify the intestinal microbiome of mammals, which directly influences host immunity[113]. This has also been observed with *H. bakeri*, as it was demonstrated that *H. bakeri* significantly raised *Lactobacillus* species abundance in the duodenum[154] and ileum[155] of infected mice and this correlated positively with a heightened

Treg response. Interestingly, it was also observed that *Lactobacillus* species abundance positively correlated with susceptibility to *H. bakeri*, as C57BL/6 mice, which are highly susceptible to *H. bakeri* infection, had raised *Lactobacillus* species abundance in response to infection, but this was not observed in BALB/c mice, which are relatively resistant[154]. Further, administration of the rodent commensal species, *Lactobacillus taiwanensis,* to BALB/c mice elevated regulatory T cells and resulted in greater helminth establishment[154]. Thus it is understood that a tripartite interaction exists between the host immune system, microbiota and *H. bakeri*.

2.2.5. Effect of maternal *Heligmosomoides bakeri* infection on offspring development

Our lab has explored the consequences of maternal GI nematode infection on fetal and neonatal development using timed pregnant mice and *H. bakeri,* as the experimental model. The CD-1 outbred mouse strain and trickle infection protocol were selected in an attempt to mimic what may be observed in a natural population, where individual variability between mice is high, and exposure to parasites is frequent [140,156].

Of relevance, it was found that *H. bakeri* infection during pregnancy and lactation increased circulating Th2 cytokines (IL-4, IL-5, IL-10 and IL-13) in the dam[8]. This increase in Th2 cytokines was similarly observed by other labs, as well as a decrease in Th1 (IFN-γ) and Th17 (IL-17) cytokines, and an increase in parasite-specific IgG1[7,9], which was transferred (via nursing) to pre-weaned 10-day old neonates, protecting them against *H. bakeri* [7]. It was also observed by our lab and another, that maternal *H. bakeri* infection altered the maternal microbiome as well as the microbiome of uninfected neonates[105] and three-month old offspring, and this was associated with increased production of short-chain fatty acids (SCFAs) [9]. It has also been observed that maternal infection impacts the growth of offspring as fetal and juvenile (PD 14 and 21) offspring of *H. bakeri* infected dams had lower crown-rump length[18-20], and juvenile offspring also had lower body mass[20]. As the placenta plays an important role in regulating fetal growth [157], a microarray-based study was performed on placental gene expression in response to an *H. bakeri* infection and it was observed that this maternal infection altered placental gene expression (214 genes up-regulated and 109 down-regulated) [158]. As the placenta also plays an important role in modulating fetal brain development [159-161], Starr et

al.[158] raised the idea that this maternal infection may influence neurodevelopment of the developing fetus. Our lab explored whether fetal or neonatal brain genes were differentially expressed in response to this maternal infection. It was found that 96 genes (88 up-regulated and eight down-regulated) were differentially expressed in the fetal brain [162], and while brain gene expression was largely unaffected two days after birth, a dramatic response was observed in seven-day old male neonates as maternal infection upregulated 2751 genes and downregulated 2985 genes[3]. Surprisingly, the neonatal gene expression data on day seven indicated that this maternal infection may actually have a beneficial influence on offspring cognition[3].

Taken together, these results clearly demonstrate an impact of this GI nematode infection during pregnancy and lactation on the development of uninfected offspring. Given the influence of the immune system and microbiome on brain development and function, both of which may be altered by a maternal *H. bakeri* infection, it is hypothesized that maternal GI nematode infection may influence neurodevelopment, having important long-term consequences for cognitive function.

2.3. Brain development in mice

Brain development is a complex organization of processes under genetic, environmental, and immune regulation, which begins during the intrauterine period and continues postnatally in rodents until three-months of age when brain maturation is completed[163].

2.3.1. Neurons and synapses

Neurogenesis (the generation of new neurons) begins at embryonic day (ED) 9.5 and is completed by ED 15 [164,165]. Synapses between neurons begin to form shortly before birth (ED 18), and peak in number during postnatal week two [166,167]. This increase in synaptic density is followed by an activity-dependent elimination or pruning of excess synapses which contributes to plasticity and is a mechanism by which the brain circuitry is refined and more complex neural networks are established[167,168]. This allows for more efficient processing of cognitive, learning and memory functions[167,168]. Refinement and maturation of neural circuitries relies on activity-dependent plasticity, such as long-term potentiation (LTP) and long-term depression (LTD), which are the persistent strengthening (LTP) or weakening (LTD) of synapses that produce a

long-lasting increase or decrease in signal transmission between two neurons[168,169]. This critical period of activity-dependent plasticity during development is completed by four to five weeks of age[5,167]. However, these processes of brain plasticity that are involved in the maturation of neural circuitries also occur in the developed brain, where, for example, LTP and neurogenesis participate in functional remodeling of neural networks during the formation of memories[170].

2.3.2. Glial cells

Gliogenesis (the generation of non-neuronal glial cells) occurs from late embryonic development into early postnatal development, with adult numbers being reached by one month of age [167,171], at which point, they comprise 70% of all brain cells[172]. The three types of glial cells in the mammalian central nervous system (CNS) are oligodendrocytes, astrocytes, and microglia. Oligodendrocytes and astrocytes are embryologically derived from the neuroepithelium, and first appear in the mouse brain around ED 12[173]. In contrast, microglia are derived from the yolk sac (i.e., extra-embryonic) mesoderm, more specifically from the hematopoietic lineage that gives rise to monocytes and macrophages, and they migrate into the mouse CNS beginning at approximately ED 10.5[173]. During this developmental period, glial cells go through a rapid period of maturation which involves morphological and functional changes, during which, the appropriate glial–neuronal interactions are instituted[167].

Due to their close relationship with neurons, glial cells play a critical role in neurodevelopment and brain function. Oligodendrocytes generate and maintain myelin sheaths around axons, necessary for the rapid saltatory propagation of action potentials[174], with onset of developmental myelination occurring during postnatal week two, and peaking during week three[167]. Astrocytes are involved in the maintenance and regulation of neuronal function, synaptogenesis, neurotransmitter cycling, metabolic support of neurons, modulation of synaptic transmission, maintenance of the blood–brain barrier, and are crucial regulators of neuroimmune responses[175-177]. Microglia are the primary innate immune cells of the CNS, but they also support myelination, neurogenesis, induce cell death or survival, and participate in synaptic pruning, formation and maturation, to ensure appropriate neuronal connections are made during brain development[178,179]. For instance, microglia support cell genesis and health through the synthesis and release of insulin-like growth factor 1(IGF1) and a variety of cytokines, and they

stimulate dendritic spine and synapse formation via the release of brain-derived neurotrophic factor (BDNF), which is important for learning and memory functions[180,181].

2.3.3. Hippocampus, long-term potentiation and spatial memory development

Since the 1950s, the hippocampus has been extensively studied in rodents as a powerful circuit model for learning about memory formation and spatial navigation[182], and it is now understood that the dorsal hippocampus is associated with spatial navigation, learning and memory, and the ventral hippocampus is involved in emotional behavior [183]. The rodent hippocampus develops postnatally and contains cornu ammonis (CA) fields (CA1-CA3) and dentate gyrus (DG)[184]. The hippocampal system has a trisynaptic loop pathway which includes the perforant pathway (the projection from the entorhinal cortex [EC] to granule cells in the DG), the mossy fibers (the projection from the DG to CA3 pyramidal cells), and the Schaffer collaterals (the projection from CA3 to CA1 pyramidal cells)[185]. Each synapse in the loop is excitatory and information flow is unidirectional[185].

The hippocampus is a highly plastic structure, with LTP of glutamatergic synapses being very prominent, and a leading candidate for the neural substrate underlying learning and memory[4,65]. Due to its predictable organization and readily inducible LTP, the CA1 region of the hippocampus has become the most commonly studied site of mammalian LTP [186]. LTP of CA3–CA1 glutamatergic synapses is assessed by stimulating the axons (Schaffer collaterals) of the CA3 neurons with a high frequency stimulation (HFS) and recording the field excitatory postsynaptic potentials (fEPSPs) from a population of CA1 postsynaptic pyramidal cells[187,188]. In the CA1 region, LTP is induced by a post-synaptic influx of calcium through the glutamate receptor, N-methyl-d-aspartate (NMDA)[189]. Amino-3-hydroxy-5-methyl-4-isoxazole- propionic acid (AMPA)-type glutamate receptors are added to the post-synaptic cell to potentiate the synapse, and over time additional proteins are synthesized to maintain LTP[189]. Activation of NMDA receptors has been shown to be critical for activity-dependent synaptic plasticity in the hippocampus, and spatial learning and memory[190].

Behavioral and physiological evidence indicates that the hippocampus matures later in postnatal development than most brain structures involved in learning and memory[191]. LTP in

the CA1 region is detected in the mouse hippocampal slice preparation as early as 2 weeks postnatal[192], however, due to the necessary maturation in presynaptic and postsynaptic mechanisms that occurs over the next few weeks[190,191], developmental onset of enduring LTP does not occur until four to five postnatal weeks[5]. With respect to spatial cognition, signs of adult-like function does not appear until at least the end of the third postnatal week in rodents, with the age of onset of higher-order learning and memory processes, including long-term retention of memories, being delayed further [190,191,193]. Modification to NMDA and AMPA receptor composition during late postnatal development is suspected to play a role in age-related improvements in spatial navigation and memory[190,194-196].

Spatial memory is a hippocampus-dependent form of memory and is responsible for the storage and retrieval of information that is needed both to plan a route to a desired location and to remember where an object is located or where an event occurred [197-199]. Remembering where things are – object–location memory – is essential for daily-life functioning and is hippocampus-dependent[200,201]. At PD 16-17, rodents can retain object location memories for 1–10 min [1,202], but longer-term retention of a few hours does not occur until PD 21–24[1,203]. Spatial reference memory is also hippocampus-dependent and is a form of memory that represents the spatial, contextual, and factual aspects of a task that remains constant between trials[204]. This form of memory is widely studied in rodents as it provides insight into how the brain encodes, stores, and retrieves information[204]. Rodents can retain short-term reference memories by three-weeks of age, however, the ability to retain these memories long-term, does not occur until much later[2,193]. For instance, At PD 20 and 34, rodents are capable of learning the route to an escape location, and remembering this location for one-day, however, the ability to remember this escape location after three or seven days does not occur until PD 60 [2].

The hippocampus contains a large number of cytokine receptors, and one of the highest densities of microglia in the brain[6]. As this brain region is responsible for spatial learning and memory[50], these cognitive processes are strongly influenced by immune molecules.

2.3.4. Cytokines and brain development and spatial memory

Immune molecules are critical for normal brain development[6,205]. During brain development and normal brain function, microglia, astrocytes, oligodendrocytes and neurons

produce, and respond to, cytokines and chemokines[206]. A large number of cytokines, including TGF-β, TNF-α, and IL-1β, have been characterized for their importance in many neurodevelopmental processes including neurogenesis, neuronal and glial cell migration, proliferation, differentiation, and synaptic maturation and pruning [6] [205]. In addition to their involvement in neurodevelopmental processes, cytokines are also known to influence hippocampal plasticity, and thus ultimately regulate spatial learning and memory[207]. For instance, TNF-α is important for activity-dependent synaptic scaling within the hippocampus[208], and both IL-1β and IL-4 are necessary for the maintenance of hippocampal LTP and hippocampal-dependent learning and memory[26,209]. Further, altering the balance of these cytokines can influence cognitive function, as high levels of IL-4 enhance hippocampal LTP[24], neurogenesis[12] and spatial memory[25], likely via the promotion of BDNF production by astrocytes and microglia[12,22,210,211], while increased expression of IL-1β reduces hippocampal BDNF levels and impairs hippocampal LTP, learning and memory [6,51-53]. Thus, due to the critical roles that immune molecules and glial cells play in brain development and function, environmental factors that cause an imbalance in the neuroimmune system can result in lasting changes in CNS structure and function[43,44].

2.4. Maternal infection and perinatal brain development

As brain development is a very complex and sensitive process, environmental and maternal factors during the perinatal period, including exercise[212-215], infection[43,44], nutrition[124-127], toxins[216] or stress[217], can significantly alter the developmental trajectory and function of cells, neural circuits, and associated behavioral outcomes [6]. Among these factors, maternal infection is particularly relevant to my thesis.

2.4.1. Maternal viral and bacterial infections and neurodevelopment

Numerous epidemiological reports suggest an association between neurodevelopmental disorders, like ASD and schizophrenia, and prenatal exposure to viral or bacterial pathogens [39,40,218]. Rodent models have helped to further establish this relationship and it is now understood that it is the maternal immune response, not a specific pathogen, which is a risk factor for neurodevelopmental disorders[42]. In this context, numerous studies have induced

maternal immune activation (MIA) in rodents, mostly in mice, using a range of inflammatory stimuli including lipopolysaccharide (LPS), a cell wall component from Gram negative bacteria, and polyinosinic:polycytidylic acid (poly IC), a synthetic double-stranded RNA analog, to mimic bacterial and viral infections, respectively[44].

Using bacterial and viral pathogens or their mimics, a strong Th1 and Th17 immune response is observed in the mother, resulting in an increase in circulating cytokines, particularly IL-1β, IL-6, TNF-α and IL-17A [44,47]. This inflammatory response in the mother has been associated with cytokine changes in the placenta, with placental levels of IL-1β, TNF-α, IL-6 and IL-17a increasing[47,219-223]. These MIA models have also been associated with an increase in these pro-inflammatory cytokines in the fetal and neonatal brain [45-48,60,224-226], and peripheral serum [225,227]. Of note, IL-6[46,220,226,228] and IL-17A[47,229] in particular have been identified as the main cytokines that appear to be critical for mediating behavioral abnormalities in MIA offspring, with the role of IL-17A in MIA models leading to the discovery that the maternal gut microbiome is a critical factor in the development of brain and behavioral abnormalities in MIA offspring[229]. More specifically, it was observed that IL-17A is elevated in the maternal serum if segmented filamentous bacteria, which are major contributors to the differentiation of Th17 cells, are present in the maternal microbiota. Behavioral abnormalities were not observed in offspring of immune challenged mothers that lacked segmented filamentous bacteria[229].

Microglia and astrocytes are highly plastic cells which take on a wide repertoire of states and functions[230], and in response to inflammatory cytokines, such IL-1β, TNF-α, IL-6 and IL-17A, they can become activated and release inflammatory mediators which drives neuroinflammation[11,12,211,231] and can be detrimental to brain development and function. In MIA models, pro-inflammatory cytokines are associated with increased microglia[48,54,58,223,232-240] and astrocyte[48,226,241,242] density and activation in offspring brains, which can drive neuroinflammation and contribute to oligodendrocyte death and hypomyelination[241,243-246], impaired hippocampal neurogenesis [58,247-250], dysregulation of neurotransmitter systems, including the dopamine [251,252], serotonin [253-255], glutamate [54,256-258] and Gamma-aminobutyric acid (GABA) systems [259-261], reduced expression of proteins involved in synaptic structure, function, and plasticity, like BDNF[240,262], and impaired LTP[56,60,250,256]. These brain changes are associated with a number of behavioural abnormalities associated with ASD and schizophrenia, such as deficits in social interaction[43,263,264], enhanced anxiety-like behaviour[265], repetitive

behaviour[263,264,266], deficits in prepulse inhibition (i.e. sensorimotor gating) [225,237,267], and cognitive impairments, such as impaired spatial learning and memory[55-57,59,60,256,268]. It is important to note that a number of factors can contribute to differences in offspring outcomes in these MIA models, including species (rat or mouse) and strain, immunogen used, pup sex and age during testing, brain regions studied, and timing of immune insult[43,269].

A number of therapeutic strategies have been tested in these models, with anti-inflammatory cytokines and drugs being effective in treating phenotypes of MIA offspring. For instance, treatment with N-acetylcysteine[56] or minocycline[58,237,239], which have anti-inflammatory properties, was shown to reduce inflammatory cytokines, microglia activation and density, restore neurogenesis, prevent impaired hippocampal LTP, and rescue behaviors such as impaired social interaction and spatial memory. Further, treatment with the anti-inflammatory cytokine, IL-10, reduced inflammatory cytokines, suppressed microglia and astrocyte activation, and rescued oligodendrocyte death, hypomyelination, and the impaired hippocampal LTP and spatial memory observed in MIA offspring[60,241]. Finally, of great interest, Bilbo et al. 2016[106] found that maternal infection with the tapeworm, *Hymenolepsis diminuta*, prevented an increase in hippocampal IL-1β expression in neonates, which had been infected with *Escherichia coli.* This study provided the first experimental evidence that maternal GI helminth infection protects offspring from harmful neuroinflammatory responses. Of note, however, the influence of maternal GI helminth infection on the neuroimmune environment and cognitive function of the uninfected offspring was not assessed in this study, and remains to be a large research gap.

2.4.2. Maternal *Heligmosomoides bakeri* infection and neurodevelopment

While infections that induce pro-inflammatory responses in the mother are detrimental to neurodevelopment [44,47], it is unknown how an immunoregulatory inducing GI nematode infection may influence the brain development and function of offspring. In response to a maternal *H. bakeri* infection, brain gene expression data from one-week old male pups indicated up-regulation of five key interacting pathways associated with LTP[3]. Furthermore, consistent with the heightened Th2/Treg immune response in *H. bakeri* infected dams[8,9], maternal infection upregulated Th2/Treg pathways and genes encoding the anti-inflammatory cytokines IL-4 and TGF-β, along with the Treg biomarker, Foxp3, in the offspring brains[3,10]. Additionally,

Th1/Th17 pathways and the pro-inflammatory cytokine, IL-1β, were down-regulated [3,10]. Considering TGF-β and IL-4 typically activate microglia and astrocyte phenotypes that are associated with immune regulation, neuroprotection, tissue repair, and production of BDNF[11-13,22,270-273], and are known to have measurable downstream effects on LTP and cognition[22-26], these observations indicate that maternal *H. bakeri* infection may promote a Th2/Treg biased neuroimmune environment, which may be beneficial to cognitive processes in the growing offspring.

2.5 Summary

The developing brain is vulnerable to maternal bacterial and viral infections that induce neuroinflammation in the offspring, leading to irreversible neurodevelopmental defects and associated behavioural and cognitive impairments[44,47]. In contrast, our lab has previously found that maternal infection with the murine intestinal nematode, *H. bakeri*, which induces an immunoregulatory response in the infected mother[8,9], may be beneficial to offspring neurodevelopment, with downstream consequences for cognitive function[3]. Brain gene expression in seven-day old neonatal male offspring of *H. bakeri* infected dams revealed up-regulation of LTP and Th2/Treg pathways, including genes for the potent immunoregulatory cytokines IL-4 and TGF-β[3,10]. This indicated that the Th2/Treg immune response in the *H. bakeri* infected mother may be transmitted to their offspring and mimicked in their brain, which in turn may be positively associated with offspring neurodevelopment, given that TGF- β and IL-4 are known to have measurable downstream effects on LTP and cognition [22-26]. As spatial memory in rodents is widely regarded as dependent on hippocampal LTP[4,65], I hypothesized that this maternal GI nematode infection may enhance the spatial memory of uninfected offspring due to enhanced hippocampal LTP, which is mediated by a Th2/Treg biased neuroimmune environment.

Therefore, in the subsequent chapters of my thesis, I examine the consequences of maternal GI nematode infection on offspring spatial memory, hippocampal long-term potentiation, gene expression and neuroimmunity, and resistance to direct infection as an index of peripheral immunity.

NOTE: References for Chapter II - Literature Review can be found in the "Master List of References for All Non-Manuscript Sections" located at the end of the document.

Chapter III - Maternal gastrointestinal nematode infection enhances spatial memory of uninfected juvenile mouse pups

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Abstract

The developing brain is particularly vulnerable to factors including maternal infection during pregnancy. Establishment of neural networks critical for memory and cognition begins during the perinatal period, when *Heligmosomoides bakeri*, a gastrointestinal (GI) nematode restricted to the maternal mouse intestine, has been shown to upregulate expression of long-term potentiation genes in the young rodent pup brain. We explored the impact of maternal infection during pregnancy and early lactation on the spatial behavior of uninfected male and female juvenile mice. Pre-weaned pups of *H. bakeri* infected dams exhibited less exploratory behaviour compared to pups of uninfected dams on postnatal day (PD) 16 but not PD 17, possibly reflecting a transient fear of an unfamiliar environment and/or a brief neurodevelopmental delay. Our two spatial memory tests show for the first time an enhancement of spatial memory in response to maternal nematode infection regardless of pup sex. At PD 17, pups of infected dams expressed object location memories after 3 hours in the Object Location Test whereas offspring of uninfected mothers did not. In addition, at PD 34, juveniles of infected mothers retained their ability to find the escape hole in the Barnes Maze Test for one week whereas offspring from uninfected mothers did not. This finding is even more striking given that spatial memory was positively associated with pup length, yet this maternal infection impaired linear growth of pups. Thus, the positive impact of maternal infection on spatial memory countered any impairment associated with the shorter length of the pups. Overall, these novel findings indicate that a maternal GI nematode infection during pregnancy and lactation positively influences the spatial memory of uninfected juvenile offspring with potential fitness implications for the next generation.

Introduction

In most natural environments, terrestrial mammal populations harbour gastrointestinal (GI) helminths that often live as adults for prolonged periods [1]. Parasites have been found to have profound effects on host behaviour and cognition[2,3], and the impact of GI helminths on cognitive function has been debated for many years [4]. Rodent studies in controlled laboratory settings showed that the GI helminths *Nippostrongylus brasiliensis*[4], *Heligmosomoides polygyrus*[5] and *Ancylostoma ceylanicum*[6] impaired spatial learning and memory of the infected host, that *Hymenolepsis diminuta* improved spatial memory of the infected host[7], and that *Strongyloides ratti* did not influence spatial learning or memory of the infected host[8]. Spatial memory is an important aspect of cognitive function that is needed both to plan a route to a desired location and to remember where an object is located or where an event occurred[9]. For mammals, mate location, foraging, predator avoidance and territorial defence are all dependent on spatial memory, and it is therefore an essential aspect of survival[10]. Impairment of spatial memory may thus reduce fitness of the infected host, while an enhancement would assumingly be beneficial. Previous studies have focused on the infected host, but it is unknown whether maternal GI helminth infection influences the spatial behaviour of the next generation. This is surprising as pregnancy increases the risk of helminth infection[11,12], and brain development has been shown to be particularly vulnerable to factors such as maternal stress, malnutrition, and infection during pregnancy[13-15].

Brain development is an extremely complex and sensitive process which begins during the intrauterine period and continues postnatally in rodents until three-months of age when brain maturation is completed[16]. Active exploration of space begins in the second week of life[17], but the refinement and maturation of neural circuitries necessary for efficient processing of spatial cognition only occurs at three to four weeks of age[17] when rodents can form and retain spatial memories for the location of objects and the route to a specific location[18,19]. During this developmental period, the persistent strengthening of synapses that produces a long-lasting increase in signal transmission between two neurons is an important and necessary process for spatial memory formation[20]. This process is called long-term potentiation (LTP), and occurs in all excitatory pathways in the hippocampus[20], the part of the mammalian forebrain network that is necessary for spatial cognition [21]. As functional hippocampal memory is sensitive

to perturbation[22], any stressors that occur during the neurodevelopmental period, including maternal infection, can have long-lasting consequences on brain function and behaviour. For example, exposure of pregnant rodents to *Escherichia coli*[23,24] or Influenza virus[25] reduced the induction of hippocampal LTP in offspring, and impaired their spatial exploration, learning and memory.

Among the behavioral tasks designed to assess spatial behaviours in rodents[9], the Open Field Test (OFT) is commonly used to assess exploratory behaviour. Spontaneous exploration is first detectable between post-natal day (PD) 16-19 and a lack of exploration may indicate anxiety[17,26]. For spatial memory assessment in young mice, the Object Location Test (OLT) is minimally stressful and relies on an animal's intrinsic preference for novelty[27]. This test is hippocampus-dependent and assesses the ability of rodents to recognize that the location of an object has changed between a training and test trial, evidenced by an increase in investigation of the object after it has been displaced[27,28]. Young mice (PD 16-17) can retain object location memories for 1-10 minutes[18,29] but longer-term retention does not occur until PD 21-24[18,30]. The Barnes Maze Test (BMT) is another spatial test that avoids the use of strong aversive stimuli, and assesses hippocampus-dependent spatial reference memories formed over repeated trials in an unchanging environment[9]. This test assesses the ability of rodents to learn and recall the location of an escape box which is located under one of 20 holes around the perimeter of a platform[9]. The BMT can assess both short-term spatial reference memory one day after the training phase and long-term spatial reference memory one week later[31]. Rodents are capable of learning the route to an escape location at PD 21-23[17,19,32,33], but the ability to retain long-term memories for this location does not normally occur until adulthood[32,33].

In examining the impact of maternal infection on offspring cognition, it is important to recognize that both offspring sex[34-37] and body length[38-41] may influence the results. The ability of male rodents to outperform females in spatial tasks may be linked to hormonal influences[37,42], or the size of the hippocampus[43]. Height is a strong indicator of brain size and cognitive function, including memory, in humans[38-41]. In rodents, length has shown to be associated with brain size[44], which is associated with cognition[45]. Although a relationship between length and cognitive performance has not been documented in rodents, it may be important to control for

length when measures of brain size are not available especially as maternal infections can impair offspring linear growth[46,47].

Due to the gap in research surrounding the influence of a maternal GI helminth infection on the neurodevelopment of offspring, a recent study has explored the consequences of maternal infection with the GI nematode, *Heligomosomoides bakeri* (also referred to as *Heligomosomoides polygyrus* and previously known as *Nematospiroides dubius*), on neonatal brain development[48]. *H. bakeri* is common in wild mouse populations with a prevalence as high as 86%[49], and is a commonly used laboratory model[50]. This strictly intestinal parasite has a direct lifecycle whereby eggs shed in the feces of the infected mouse hatch in the external environment and undergo two molts to become infective third stage larvae (L_3) within 7 days. Infective L_3 are then ingested and penetrate the submucosa of the small intestine before returning to the intestinal lumen as adult worms where they mate, and female worms release eggs[50]. Infected mice mount a strong type 2 (Th2) immune response against the parasite, however, adult worms are capable of stimulating an immunoregulatory network (Treg) which facilitates parasite survival[50,51]. *H. bakeri* infection of pregnant and lactating mice has been shown to alter gene expression in fetal[52] and neonatal brains[48], and to up-regulate five key interacting pathways associated with LTP in uninfected one-week old male pups[48]. These findings raise the intriguing hypothesis that a maternal *H. bakeri* infection may improve synaptic plasticity, cognition and memory of the next generation. The goal of this study was to explore the influence of maternal *H. bakeri* infection on the spatial behaviour of their uninfected pre-weaned and juvenile male and female offspring.

Results

This study assessed the influence of maternal *H. bakeri* infection on the spatial behaviour of uninfected male and female juvenile offspring. Outbred CD-1 mice were infected repeatedly or sham infected during pregnancy and lactation and litters from 8 uninfected and 8 *H. bakeri* infected dams were used to explore spatial exploration, learning and memory as well as litter size, crown-rump length, and body mass.

Impact of Maternal Infection on Litter Size

There was no significant effect of maternal infection on litter size (uninfected: 12.4 ± 1.12; infected : 12.9 ± 0.61; P = 0.7).

Impact of Maternal Infection and Offspring Sex on Pup Crown-rump Length and Body Mass

Pups born to infected dams had shorter length and lower mass than pups of uninfected dams, at both PD 15 and 21 (all P values < 0.0001, Supplementary Fig. 1). In addition, male pup length and mass were larger than female pups at both PD 15 and 21 (all P values < 0.005, Supplementary Fig. 1).

Impact of Maternal Infection on Offspring Spatial Behavior

Early exploratory behavior

In the OFT, pups of infected dams exhibited less spatial exploration at PD 16 than pups of uninfected dams as evidenced by lower *total path traveled* (P = 0.01), *mean velocity* (P = 0.01), and *time in center zone* (P = 0.015) and a greater *time without movement* (P = 0.006) (Table 1). There were no sex effects (all P values > 0.15, data not shown).

Object location memory

There was no bias in exploration of Objects 1 and 2 during the training phase (P = 0.40). Furthermore, maternal infection did not affect the *total path traveled, mean velocity, time without movement*, *object investigation time* (Table 2) or *% investigation time of mobile object* (Fig. 1) during the training trial or during the test trial. Additionally, there were no sex effects (all P values > 0.27, data not shown).

However, when using our derivative variables to compare object location memory between training and test trials, we found that maternal infection improved pup memory. Comparing the *% investigation time of mobile object* between training and test trials, pups born to infected dams remembered object locations and explored the moved object significantly more

during the test trial compared to the training trial (P = 0.019) (Fig. 1) whereas pups of uninfected dams spent a similar % *investigation time of mobile object* in both the training and test trial (P = 0.74) indicating that they had not retained object location memory after a 3 hr period (Fig. 1). This was also reflected in the *change in % investigation time of mobile object* (Fig. 2). Pups of infected dams increased their investigation of the mobile object during the test trial whereas pups of uninfected dams did not, and the difference between these two groups was significant (P = 0.027), providing further evidence that pups of infected dams were able to recall object location memory for 3 hours. These findings were not affected by offspring sex (all P values > 0.28, data not shown), but *change in % investigation time of mobile object* was positively associated with pup length (P = 0.041, data not shown), independent of maternal infection.

Spatial learning

In the BMT, regardless of maternal infection or offspring sex, pups learned the location of the escape hole on the first training day as indicated by a decrease in the average *latency* (P < 0.0001; Fig. 3a), *path length* (P < 0.0001; Fig. 3b), and *errors* (P < 0.0001; Fig. 3c) between training days 1 and 2. Thereafter, values remained low. Neither maternal infection nor offspring sex influenced *mean velocity* (all P values > 0.27, data not shown).

Short and long-term spatial reference memory

Neither maternal infection (all P values > 0.34, Fig 4a-c) nor offspring sex (all P values > 0.47, data not shown) altered short-term spatial reference memory in probe trial 1. However, regardless of sex, offspring born to infected dams had enhanced long-term spatial reference memory (Fig. 4) as assessed in probe trial 2. Offspring of infected dams had lower *latency* (P = 0.0044; Fig. 4a), *path length* (P = 0.0067; Fig. 4b), and fewer *errors* (P = 0.0031; Fig. 4c) in finding the escape hole than offspring of uninfected dams. Furthermore, when controlling for individual performance in probe trial 1, offspring of infected dams retained their memory over the one-week interval whereas the performance of offspring of uninfected dams declined strongly as shown by the positive *change in latency* (P = 0.0067; Fig. 4d), *path length* (P = 0.015; Fig. 4e), and *errors* (P = 0.01; Fig. 4f). Of note, while findings were not influenced by offspring sex (all P values > 0.1, data not shown), independent of maternal infection, long-term

spatial reference memory was positively associated with offspring length (all P values < 0.0015, data not shown). Thus, despite offspring of infected dams being significantly shorter, they outperformed offspring of uninfected dams.

Discussion

Using a nematode parasite that remains in the maternal intestine, we tested our hypothesis that maternal infection during pregnancy and lactation would positively influence the spatial behavior of pre-weaned and juvenile uninfected male and female offspring. We report for the first time that PD 16 offspring of *H. bakeri* infected dams exhibit less exploratory behaviour compared to pups of uninfected dams, possibly reflecting transient fear of an unfamiliar environment and/or a brief developmental delay. However, in response to maternal infection, PD 17 offspring exhibited better retention of object location memory and at PD 34 they had enhanced long-term spatial reference memory. These novel findings indicate that a maternal GI nematode infection during pregnancy and lactation positively influences the spatial memory of uninfected juvenile mice, despite the negative impact of maternal infection on the linear growth of the pup.

Findings from the OFT indicate that on first introduction to an open arena, offspring of *H. bakeri* infected mothers explored less compared to offspring of uninfected mothers, raising the possibility of a developmental delay and/or heightened fear or anxiety[17,26]. As spontaneous exploration in an open field is first detectable between PD 16-19[17,26], it is possible that some component of neurodevelopment is delayed at PD 16 in response to maternal infection, which may have negative consequences for the offspring. However, this lower exploration was not observed one day later when these pups were placed in the open field with two novel objects, suggesting that if a developmental delay did occur, it was brief, and may not have had consequences for the growing pup. These findings from the OFT are similar to other maternal infection studies as exposure of pregnant mice to *E. coli*[53] or influenza virus[25] resulted in heightened anxiety-like behaviours albeit in adolescent (5 week old)[53] and adult (9 month old)[25] offspring, indicated by less exploratory behavior in an OFT. While a heightened fear/anxiety response can be considered a negative attribute, under some circumstances it can be

advantageous to the host[3]. Fear and anxiety act as a response to danger or threat[54], thus when exposed to an unknown environment, mammals typically freeze as it is more difficult for a predator to observe a non-moving animal[26]. Considering that wild rodents are exposed to a number of natural predators, the lower exploration in the open field arena in response to maternal infection may indicate more caution when placed in an unknown and potentially dangerous environment which could actually be beneficial to survival.

The ability to recognize and remember the spatial characteristics of the environment, such as the location of objects, is an important component of spatial cognition[9,19]. This typically begins in 16-17 day old rodents with memory lasting only for a few minutes[18,29], but for a few hours in 21-24 day old subjects[18,30]. Thus, our observation that PD 17 pups of uninfected mothers were unable to detect object rearrangement after a three hour period was consistent with the literature and suggests a normal immaturity in recalling spatial information at PD 17[55]. However, despite their young age we found that PD 17 pups of *H. bakeri* infected mothers were able to retain object location memories for three hours, as evidenced by a significant increase in investigation of an object after it had been moved. This finding is in contrast with reports that exposure of pregnant rodents to viral mimics had no influence on offspring object location memory, although the studies were done using adult offspring[56,57]. Our findings indicate that the maturational process needed to recall object location memories for three hours occurred earlier as a result of maternal *H. bakeri* infection. This is consistent with recent findings that maternal *H. bakeri* infection up-regulated expression of genes associated with LTP in brains of perinatal uninfected offspring[48] and thus may promote cognitive development.

The ability to learn the route to an escape location is detectable at PD 21 in rodents[17,19,32,33], however, long-term reference memories for an escape location in the Morris water maze do not emerge until much later[32,33]. The Morris water maze is similar to the BMT as it assesses spatial learning and reference memory by testing the ability of a subject to locate a hidden underwater platform in order to escape from water in a circular water tank[9]. When PD 20, 34 and 60 subjects were tested in a Morris water maze, all age groups were capable of learning the route to the escape location, and remembering this location for one-day[33]. However, PD 20 and 34 rodents were not yet capable of retaining long-term reference memories for a one-week period whereas PD 60 subjects were[33]. We assessed spatial learning over four days from PD 23-

26, followed by short-term reference memory one day later at PD 27 and finally long-term reference memory one-week later at PD 34. Maternal infection had no impact on spatial learning or short-term reference memory, but long-term reference memory was enhanced as a result of this maternal infection. The ability of the juvenile control pups to learn the location of the escape box and recall this location after one day but not one week was consistent with studies using the Morris water maze[32,33]. Unlike control pups, offspring from infected mothers were capable of retaining long-term reference memories for a week as they performed equally well after the one-week delay, compared with the one-day delay. These findings are in the opposite direction to reports from maternal *E. coli* infection models where exposure of pregnant rodents impaired offspring spatial learning and short and long-term reference memory in the Morris water maze[23,24,35]. Overall, our findings reinforce our observation from the OLT that the maturational processes required for the retention of spatial memories occur earlier as a result of this maternal infection and leads us to speculate that maternal *H. bakeri* infection may increase the fitness of the next generation.

Some evidence of sex dependent differences in spatial learning and memory of offspring has been reported in response to prenatal infection mimics whereby molecules of pathogens are injected into the pregnant dam[34-36]. For example, exposure of pregnant rats to *E. coli* lipopolysaccharide (LPS) impaired spatial learning and reference memory in the Morris water maze in 28-day-old male but not female offspring[35]. The underlying mechanisms are unknown, although sex hormones might play a role[35,37,42]. Other studies have shown no impact of offspring sex on spatial behaviour in response to prenatal infection mimics[58,59]. Our results using a direct nematode infection of pregnant mice are consistent with these latter studies in that offspring sex did not affect spatial exploration by offspring in the OFT, their ability to retain object location memories in the OLT, or to learn or remember the escape location in the BMT. Similarly, in the absence of maternal stress, no difference in the spatial behaviour and memory performance was observed between male and female pre-weaned (PD 17-18) CD-1 mice and rats in an OFT and OLT[18,55], nor between juvenile (PD 22) male and female mice in a Morris water maze test[60].

The observed impact of maternal infection on spatial learning and memory of their pups could be an indirect consequence of infection-induced nutrient deficiencies but evidence from the literature suggests that this is unlikely. Unlike hookworms that feed on blood and can lead to

iron deficiency anemia and protein deficiency when in high numbers[61], adult *H. bakeri* feed on the epithelial cell layer of the small intestine and are not typically associated with blood loss[62]. Despite lower maternal food intake in response to *H. bakeri* infection during pregnancy[63], we found no impact of infection on maternal body mass during pregnancy or lactation or on the date of delivery or litter size, all of which would be expected consequences of maternal malnutrition[46,64]. Furthermore, total serum protein concentrations have been shown to be higher in *H. bakeri* infected dams at day 20 of lactation[65]. In the absence of evidence of nutrient deficiencies in pups of *H. bakeri* infected dams and knowing that nutrient deficiencies would be expected to impair not improve spatial memory[66-70], it is unlikely that nutrient deficiencies account for the improved memory of pups in response to maternal infection.

Maternal *H. bakeri* infection is known to impair fetal[12] and offspring[46] linear growth as observed in our study and this impaired growth could have impacted spatial memory. In humans, height, brain size and general cognitive ability are positively correlated[38-41]. Rodent length is correlated with brain size[44] and brain size has been reported to be a strong indicator of cognitive ability, including the ability to find an escape location in laboratory mice[45]. Our novel finding that mouse length is directly correlated with spatial memory would lead to the expectation that the shorter pups of infected dams would have had impaired spatial memory. However, we found the opposite. Despite their smaller size, it is noteworthy that pups of infected dams were able to recall object locations for 3 hours in the OLT and to recall the location of the escape box in the BMT for 1 week, whereas the larger pups of uninfected dams could not.

Formation and retention of spatial memories are controlled in the hippocampus and promoted by LTP and neurogenesis[20,71]. Our observation that offspring of *H. bakeri* infected mothers have enhanced spatial memory is consistent with previous evidence that the brains of PD 7 pups of infected dams have increased expression of LTP genes as well as the ITGA3 gene[48], which may promote neurogenesis[72]. Further evidence for this hypothesis is found in physical exercise models, where exposure of mice to running enhances hippocampal neurogenesis and LTP which results in enhanced spatial memory performance in the Morris water maze[73,74]. Thus, we speculate that maternal *H. bakeri* infection is capable of enhancing hippocampal LTP and/or neurogenesis in the uninfected pup which promotes the enhanced spatial memory we observed. Further studies would be needed to explore this hypothesis.

The mechanism whereby a nematode living in the lumen of the maternal intestine could influence brain gene expression and alter cognitive processes which promote the spatial memory ability of offspring is unknown. One possibility is that the Th2/Treg immune response in the infected dam[75] induces a similar systemic response in the uninfected pup and extends to and alters the immune profile in the pup brain. Consistent with this, maternal *H. bakeri* infection up-regulated expression of Th2/Treg pathways and their associated cytokines including interleukin (IL)-4 and transforming growth factor-β (TGF-β) in the PD 7 pup brain, while down-regulating Th1 pathways and the inflammatory cytokine IL-1β[48,76]. Elevated IL-1β has been shown to impair spatial memory[77], and knock-out studies have highlighted the beneficial and critical importance of IL-4 for the formation and retention of spatial memories[78,79]. Performance of spatial tasks leads to the accumulation of IL-4 producing Th2 cells in the meninges, and deficiency of IL-4 results in severely impaired performance of spatial memory tasks[78]. IL-4 stimulates astrocytes to produce brain-derived neurotrophic factor (BDNF)[78], a key molecule for regulating cognitive processes, including LTP and neurogenesis[80,81]. Of note, in addition to up-regulating IL-4 expression[48,76], maternal *H. bakeri* infection also up-regulated BDNF expression in the brains of PD 7 neonates (unpublished data). Therefore we hypothesize that the enhanced spatial memory in the pups of infected dams is associated with a regulatory Th2/Treg neuroimmune environment which promotes LTP and neurogenesis via the production of BDNF by astrocytes. Consistent with our hypothesis that a maternal helminth infection is capable of altering the neuroimmune environment of offspring, Williamson et al.[82] found that maternal infection with *H. diminuta* blunted the normal increase in hippocampal IL-1β mRNA response to LPS injection in PD 4 offspring. Similar to *H. bakeri, H. diminuta* infects the small intestine and induces a Th2/Treg immune response[83]. Further research is needed to determine whether the Th2/Treg bias is reflected in the neuroimmune environment of the uninfected pup.

We acknowledge four limitations. First, given our hypothesis that spatial memory may emerge earlier due to this maternal infection, we needed to test pre-weaned mice in the OLT, but some of them did not meet our inclusion criterion as they did not explore either object. This was expected as pups would likely have a high level of anxiety and fear due to being separated from their mothers for the test, leading to freezing events and a complete absence of exploration of the arena and objects. Although this lowered our sample size, we had sufficient pups that did explore to be able to detect significant differences between pups of infected and uninfected mothers.

Second, we did not determine brain mass of the pups which may have been a more direct covariate for behavioural variables than body length. Third, despite the evidence for improved spatial memory, this maternal GI nematode infection may have negative (or positive) implications on other aspects of brain function and behaviour. Fourth, as our study was focused on the development of spatial cognition in young offspring, our findings cannot be extrapolated to adult mice. Future studies are needed to determine if this maternal GI nematode infection has positive long-term influences on brain development and behavior of the next generation.

To the best of our knowledge, this is the first study to assess the impact of a maternal GI nematode infection on the spatial behaviour of offspring, and to demonstrate enhanced spatial memory in pre-weaned and juvenile uninfected offspring. These findings shed light on a possible unappreciated benefit of maternal GI nematode infection and highlight a possible increase in fitness of the next generation. It would be important to determine if this behavioural impact persists as mice mature and how this maternal infection influences other aspects of offspring behaviour.

Methodology

Experimental design

We employed a 2 x 2 factorial design using *H. bakeri* infected versus uninfected dams, and their male versus female offspring.

Mice and Parasites

Of the 19 primiparous 8-week-old timed pregnant (gestation day [GD] 4) outbred CD-1 mice (Charles River Laboratories, Quebec, Canada), 16 were pregnant (84% pregnancy rate). Each dam and her litter was housed individually in a Nalgene cage (Fisher Scientific, Canada) at 21–23 °C, 40–60% relative humidity and a 12 h light and dark cycle. Mice had *ad libitum* access to a 2920X Teklad rodent diet (18% crude protein, 5% crude fat, 5% crude fiber). Within each of the seven staggered groups of dams received over 5 months, dams were randomized into uninfected and infected groups, and a total of eight dams per group were used for this study, providing an acceptable sample size based on a minimum of at least six dams per treatment

condition[84]. Using standard *H. bakeri* protocols [85], infective L_3 were obtained by fecal culture of stock parasites maintained in outbred CD-1 mice. Dams in the infected group were intubated using an oral gavage needle with 100 ± 3 L_3 suspended in 0.1 mL distilled water on GD 7, 12, 17, and PD 3, 8 and 13 (Fig. 5). Uninfected dams were intubated at the same frequency with 0.1 mL distilled water. Given that *H. bakeri* eggs released into the environment develop into infective larvae after 7 days, all cages were cleaned every 5 days to ensure offspring could not ingest infective larvae. Successful infection of dams was confirmed through faecal egg counts at weaning (PD 21), and worm counts 13-32 days after weaning (235.4 ± 45.4 worms/dam). Dams were then used for a separate experiment.

Pups were born on GD 19 or 20, litter size was recorded on PD 3, 8, 13, 15 and 21, and body mass and length from the top of the head to the base of the tail were recorded on PD 15 and 21. At PD 15, pups were sexed and given a unique identifier with a permanent marker. Pups were randomly selected to provide two male and two female pups per litter for the OFT/OLT and two male and two female pups per litter for the BMT. At weaning, pups were separated by sex and 3-4 littermates were housed per cage. After the OFT/OLT or the BMT, experimental pups were necropsied and intestines were examined for larval and adult *H. bakeri*[85.86]. This confirmed that the pups had not been accidentally infected. Pups not used for this study were assigned to a separate experiment.

Compliance with Guidelines for Research with Experimental Animals

This study (protocol #2000–4601) was approved by the McGill University Animal Care Committee according to the guidelines of the Canadian Council on Animal Care. All methods were carried out in accordance with relevant guidelines and regulations, and the study was carried out in compliance with ARRIVE guidelines (https://arriveguidelines.org).

Experimental Room and Procedures

All spatial tests were conducted in a quiet room (340 cm x 260 cm) with a floor lamp in each corner that provided dim, even illumination to minimize stress of young pups during the OFT and OLT. During the BMT, a bright over-head light was added to provide a mild negative

reinforcement. Trials were recorded using an overhead monochromatic video camera (Basler Ace monochrome) connected to a computer that was located in the back corner of the room behind a curtain. The experimenter remained behind the curtain during all recordings. Data was extracted from the videos using the Ethovision XT software (version 15). All equipment remained in the same location in the room, providing visual spatial cues.

To reduce handling anxiety, each pup in every litter was allowed to explore the palm of the experimenter for two minutes on PD 14 and 15, in their home room. Home cages were moved into the experimental room for 15-20 min acclimation prior to trials and all equipment was cleaned with 70% ethanol between trials.

Open Field Test (OFT) and Object Location Test (OLT)

The OFT/OLT arena (Maze engineers, 412 Wilmette Ave, Glenview, IL 60025, USA) (80 x 80 x 30 cm) had four opaque plexiglass compartments (40 x 40 x 30 cm) that allowed us to test the four pups per litter at the same time (Fig. 6a). An environmental cue (a large cross in colored tape) was placed on an inside wall of each compartment.

The OFT, conducted 24 hours prior to the OLT, is an important component of the OLT protocol as it allows the pups to habituate to the novel arena. It also provides information on their exploratory behaviour[28]. On PD 16, pups were introduced to a designated compartment of the arena, and their activity was recorded for 10 min [53]. A preference to stay close to the walls of the field along with freezing behavior (not moving) indicates decreased spatial exploration and increased anxiety-like behavior[26]. For the purpose of data collection, the arena was conceptually partitioned into the peripheral zone (5.86 cm from each wall, totaling 50% of the surface area), and the center zone occupying the remaining area. Four descriptive displacement variables were measured to assess activity: *total path traveled (cm), mean velocity (cm/s), time without movement (%),* and *time in center zone (%)*.

The OLT tested object location memory based on exploration of an object that had been moved to a novel location[27,28,88] between the 5 min training trial and the 5 min test trial. Pilot testing confirmed that our multi-colored metal cylindrical aerosol cans (diameter: 4 cm, height: 15 cm) were suitable objects for the OLT because young pups did not fear them or climb on, sit

on top of, or tip them over. The OLT was conducted on PD 17 using the same mice that had been habituated to the arena during the OFT on PD 16. Prior to the OLT training trial, two identical objects were positioned at designated locations within each compartment (Fig. 6a). Each pup was placed in the compartment as far as possible from both objects to avoid any position bias, and behaviour was video-recorded for 5 min after which pups were returned to their home cage. Prior to the test trial, one of the two objects (Object 2) in each compartment was moved to a novel location (Fig. 6a). A 3 hour interval between training and test was selected given that 5 week old CD-1 mice have been shown to retain object location memories after 2 hours but not after 4 hours [88], and we had hypothesized that spatial memory would be enhanced in response to maternal infection. Pups were returned to the same compartment for a 5 minute test trial. Three descriptive displacement variables were measured during both the training and test trials: the *total path traveled (cm), mean velocity (cm/s),* and *time without movement (%)*. In addition, one exploration variable was recorded for both Object 1 (stationary object) and Object 2 (mobile object): *object investigation time (s)* which measured how long a subject's nose was within a one-cm radius of the respective Object.

Pups were excluded from analysis of the OLT if they did not explore objects during either the training or test trial (6 males and 2 females from the uninfected group; 6 males and 8 females from the *H. bakeri* group).

Barnes Maze Test

The BMT procedure followed a protocol[31] that successfully tested spatial learning and short and long-term reference memory in CD-1 mice[88]. The Barnes Maze (Maze engineers, 412 Wilmette Ave, Glenview, IL 60025, USA) is an opaque circular platform (diameter: 92 cm, height: 70 cm) with 20 equally spaced holes (diameter: 5 cm) located 2 cm from the edge (Fig. 6b). In a brightly lit environment, mice naturally seek the dark enclosed area provided by the black goal box (20 × 10 × 4 cm) which was located under the same escape hole throughout all trials (Fig. 6b). From the surface of the maze, the escape hole, containing the goal box, looks identical to the other 19 holes. Mice learn the location of the goal box based on spatial cues.

The BMT was conducted on pups that had not been tested in the OFT/OLT. It involved a habitation phase of 5 min on PD 22 (Day 0), a training phase from PD 23-26 (Day 1-4), and

probe trials 1 and 2 to test short-term and long-term spatial reference memory on PD 27 (Day 5) and 34 (Day 12) respectively. Training involved four 3 min trials per day for four training days. Each of the 16 training trials began by placing a pup in an opaque starting cylinder (diameter: 10.5 cm, height: 8 cm) at the center of the platform. After 10 sec, the cylinder was removed, recording began, and the animal was allowed to freely explore the apparatus for 3 min. Once the animal entered the goal box, it was allowed to remain there for 60 sec. Mice that failed to find the goal box within 3 min were gently guided to its location and placed inside. After each of the four 3 min training trials per day, mice were returned to their home cage for 20 min. Prior to probe trials 1 and 2, the goal box was removed from the escape hole and mice explored the maze for 90 sec. No training occurred between the two probe trials.

Variables assessed during all trials were: 1) *latency (s)*, defined as time taken to the first visit (nose poke) to the escape hole; 2) *path length (cm)*, defined as distance travelled to the first visit to the escape hole; and 3) *errors*, defined as number of times a subject visited non-escape holes, before their first visit to the escape hole. *Mean velocity (cm/s)* during the training trials was used to determine if performance differences reflected motor ability that may have been influenced by pup length.

Statistical Analyses

Statistical analyses were performed in R statistical software 4.0.2[89], and figures were produced using the package ggplot2[90]. Maternal treatment condition (*H. bakeri* infected versus uninfected) and offspring sex (male versus female) were always included as fixed factors. For comparisons over time, trial was included as a fixed factor. To account for pseudoreplication, dam was a random factor in all models, and the identity of the pup was also included as a random factor for comparisons over time where we had repeated measures on pups[91]. Non-significant interactions between fixed effects were excluded from models[92]. Pup length was included as a covariate in all models of behaviour data.

Linear mixed models (LMMs) or Generalized linear mixed models (GLMMs) were built using the lmer or glmer function, respectively (lme4 package[93]), with significance assessed using the Anova function (car package[94]). Where necessary, post hoc pairwise comparisons were performed using the emmeans function (emmeans package[95]) with a Tukey correction.

Normality, independence and homogeneity of variances of mixed models were assessed using fitted residuals from the plotresid function (RVAideMemoire package[96]), and in the case of GLMMs, also using the DHARMa package[97]. Unless otherwise stated, values are presented as LSmeans ± SEM from the emmeans function. The significance level was set at 0.05.

As no pup mortality occurred, the influence of the maternal infection status on litter size was analyzed on PD 21 using a linear model (lm function[89]). LMMs were used to compare pup length and mass at PD 15 and PD 21 between experimental groups, with litter size as a covariate.

OFT/OLT: LMMs were used to assess object bias, displacement and exploration variables in the OFT and OLT. Two additional derivative variables were calculated from the OLT data to assess object location memory and both were analysed by LMM. The *% investigation time of mobile object* was used to compare the investigation of the mobile object relative to the total time spent investigating both objects in both the training and test trial, and calculated as: **[Object 2 (mobile) investigation time (s) / [Object 1 (stationary) investigation time (s) + Object 2 (mobile) investigation time (s)]] *100**. This variable ranged from 0% (only investigated Object 1) to 100% (only investigated Object 2). The *change in % investigation time of mobile object* allowed us to control results from the test trial with individual performance during the training trial, and was calculated as: **% investigation time of mobile object during the test trial - % investigation time of mobile object during the training trial**. A positive value indicated that the subject explored Object 2 more during the test trial compared to the training trial, indicating an increased investigation in the object after it had been moved.

BMT: Data in the BMT were positively skewed, and in some instances, heteroscedastic. The best distribution was assessed using the functions descdist and fitdist (package fitdistrplus[98]) and comparing model residuals for best fit. In the training phase, we used LMM with log transformations for *latency, path length* and *mean velocity*. In the probe trials, we used Gamma GLMM, with log link function, for *latency* and *path length*. The number of *errors* was a discrete and overdispersed variable, and a negative binomial GLMM, with log link function, was used for both the training and probe trials.

In addition, a set of derivative variables reflecting *change in latency, path length* and *errors* between the two probe trials was calculated by subtracting probe trial 1 values from probe trial 2 values, allowing us to control for individual performance during probe trial 1. These

derivative variables were normally distributed and homoscedastic, and LMMs were used without transformation.

Data Availability

The authors confirm that the data supporting the findings of this study are available as supplementary material. Data available under the Supplementary Information section at: https://doi.org/10.1038/s41598-022-13971-y

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Author Contributions

S.C.N. conceived and designed the study, conducted experimental work, analyzed the data, interpreted the results, and drafted the manuscript. L.F.H. provided input on the study design, assisted with experimental work and extracted data from the behavior videos using the Ethovision XT software. M.H. provided input on the study design, assisted with experimental work and provided critical suggestions that have been incorporated into the manuscript. M.E.S. provided input on the study design and data interpretation, provided critical suggestions that have been incorporated into the manuscript, and obtained funding for the research.

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Competing Interests

The authors declare no competing interests.

Tables

Table 1. Effect of maternal *H. bakeri* infection on displacement variables measured during the 10 minute Open Field Test. Pups were nested within dam and pup crown-rump length was included as a covariate. Since no significant sex differences were evident, pooled female and male data are shown. Values are LSmeans ± SEM of each outcome variable, n = 32 pups from 8 uninfected dams and n = 32 pups from 8 infected dams.

Variable	Pups of Uninfected Dam	Pups of *H. bakeri* Dam	Test Statistic & P value
Total path traveled (cm)	2586 ± 354	1191 ± 353	$x^2_1 = 6.70$; **P = 0.01**
Mean velocity (cm/s)	4.3 ± 0.6	2.0 ± 0.6	$x^2_1 = 6.69$; **P = 0.01**
Time without movement (%)	51.1 ± 5.4	74.3 ± 5.4	$x^2_1 = 7.70$; **P = 0.006**
Time in center zone (%)	11.4 ± 2.1	4.3 ± 1.6	$x^2_1 = 5.96$; **P = 0.015**

Table 2. Effect of maternal *H. bakeri* infection on displacement and exploration variables measured during the 5 minute Training and Test trials of the Object Location Test. Pups were nested within dam and pup crown-rump length was included as a covariate. Since no significant sex differences were evident, pooled female and male data are shown. Values are LSmeans ± SEM of each outcome variable, n = 24 pups from 8 uninfected dams and n = 18 pups from 7 infected dams.

Variable	Pups of Uninfected Dam	Pups of *H. bakeri* Dam	Test Statistic & P value
Training Trial			
Total path traveled (cm)	1378 ± 192	1766 ± 230	$x^2_1 = 1.37$; P = 0.24
Mean velocity (cm/s)	4.6 ± 0.6	5.9 ± 0.8	$x^2_1 = 1.36$; P = 0.24
Time without movement (%)	52.0 ± 5.6	44.5 ± 6.6	$x^2_1 = 0.61$; P = 0.44
Object 1 (stationary) investigation (s)	4.4 ± 1.1	5.8 ± 1.3	$x^2_1 = 58$; P = 0.44
Object 2 (mobile) investigation (s)	6.1 ± 1.4	4.4 ± 1.6	$x^2_1 = 0.52$; P = 0.47
Total investigation of both objects (s)	10.6 ± 1.8	10.6 ± 2.2	$x^2_1 = 0.0001$; P = 0.997
Test Trial			
Total path traveled (cm)	1391 ± 254	1564 ± 297	$x^2_1 = 0.16$; P = 0.69
Mean velocity (cm/s)	4.7 ± 0.9	5.2 ± 1.0	$x^2_1 = 0.15$; P = 0.70
Time without movement (%)	53.4 ± 5.5	49.5 ± 6.6	$x^2_1 = 0.17$; P = 0.68
Object 1 (stationary) investigation (s)	4.9 ± 1.0	3.5 ± 1.1	$x^2_1 = 0.70$; P = 0.40
Object 2 (mobile) investigation (s)	6.5 ± 2.2	6.8 ± 2.7	$x^2_1 = 0.01$; P = 0.94
Total investigation of both objects (s)	11.4 ± 2.5	10.4 ± 3.0	$x^2_1 = 0.05$; P = 0.81

Figures

Figure 1. Effect of maternal *H. bakeri* infection on % investigation of mobile object in the training trial, in the test trial, and between the training and test trials in the Object Location Test. In all models, pups were nested within dam, and pup crown-rump length was included as a covariate. Since no significant sex differences were evident, pooled female and male data are shown. Values are Means±SEM, n = 24 pups from 8 uninfected dams and n = 18 pups from 7 infected dams (ns – not significant, *$p < 0.05$).

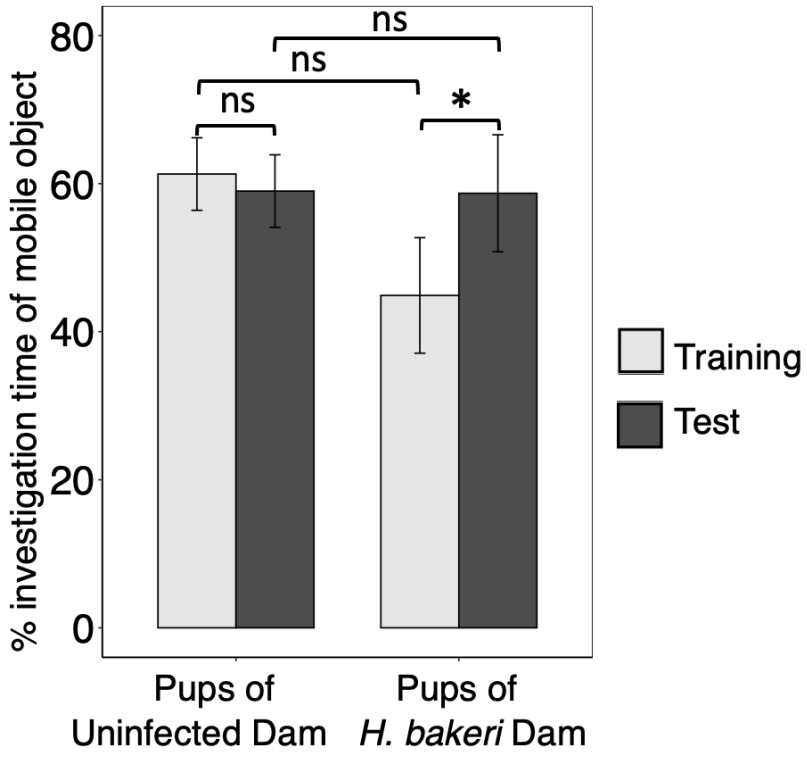


Figure 2. Effect of maternal *H. bakeri* infection on offspring change in % investigation time of mobile object between test and training trial of the Object Location Test. A positive value indicates increased investigation of the object that had been moved between the training and test trial, representing expression of object location memory. Pups were nested within dam, and pup crown-rump length was included as a covariate. Since no significant sex differences were evident, pooled female and male data are shown. Values are LSmeans±SEM, n = 24 pups from 8 uninfected dams and n = 18 pups from 7 infected dams (*$p < 0.05$).

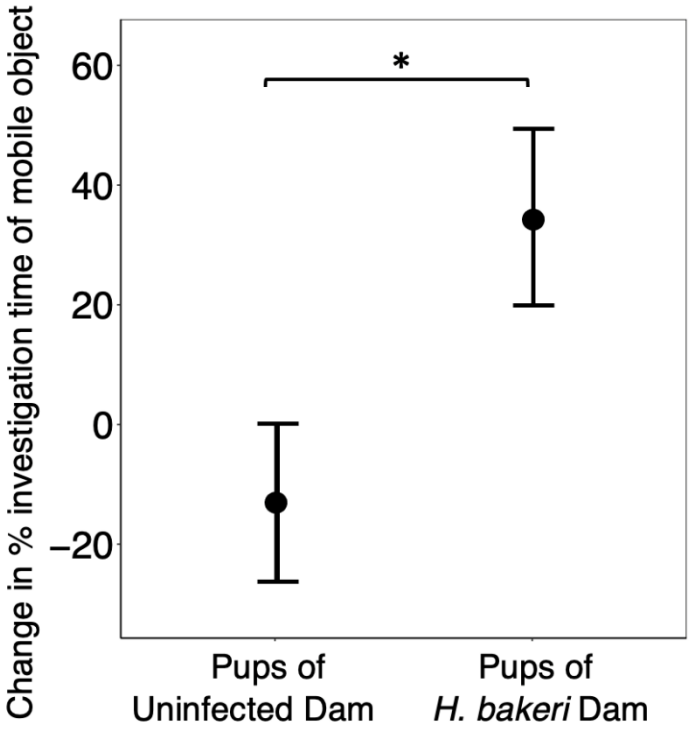


Figure 3. Spatial learning in the Barnes Maze Test over four training days. The identity of the pups was nested within dam for statistical analysis, and pup crown-rump length was included as a covariate. Since no significant differences were evident as a result of maternal treatment condition or offspring sex, pooled data is shown. Values are LSmeans ± SEM, n = 64 pups from 16 dams. (a) latency, (b) path length and (c) errors to reach the escape hole. Means without a common letter differ significantly, $p < 0.05$.

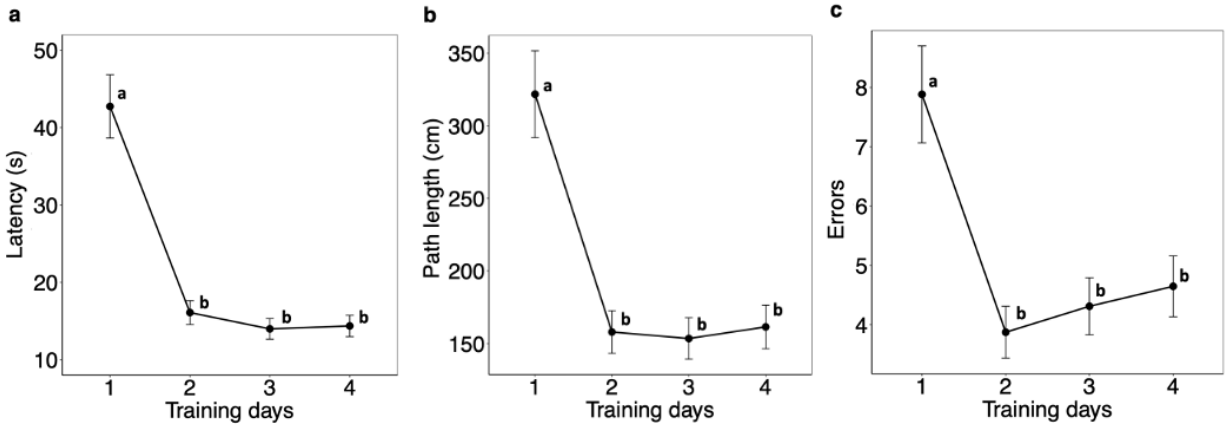


Figure 4. Effect of maternal *H. bakeri* infection on offspring short-term (probe trial 1) and long-term (probe trial 2) spatial reference memory and change in reference memory between probe trials 1 and 2 in the Barnes Maze Test. Probe trial 1 was conducted 24 hours after the last training day and probe trial 2 was conducted one-week later. Graphs A, B and C compare juveniles of uninfected vs *H. bakeri* infected dams within probe trial 1 and 2. Graphs D, E and F show the change in performance between probe trials 1 and 2 calculated by subtracting the value in probe trial 1 from the value in probe trial 2 for each mouse. A value of zero or a negative value would indicate that the subject performed as well or better during probe trial 2 compared to probe trial 1, suggesting strong memory retention. (a) latency, (b) path length, (c) errors to reach the escape hole, (d) change in latency, (e) change in path length and (f) change in errors. Pups were nested within dam, and pup crown-rump length was included as a covariate. Since no significant sex differences were evident, pooled female and male data are shown. Values are LSmeans ± SEM, n = 32 pups from 8 uninfected dams and n = 32 pups from 8 infected dams (ns – not significant, $*p < 0.05$, $**p < 0.01$).

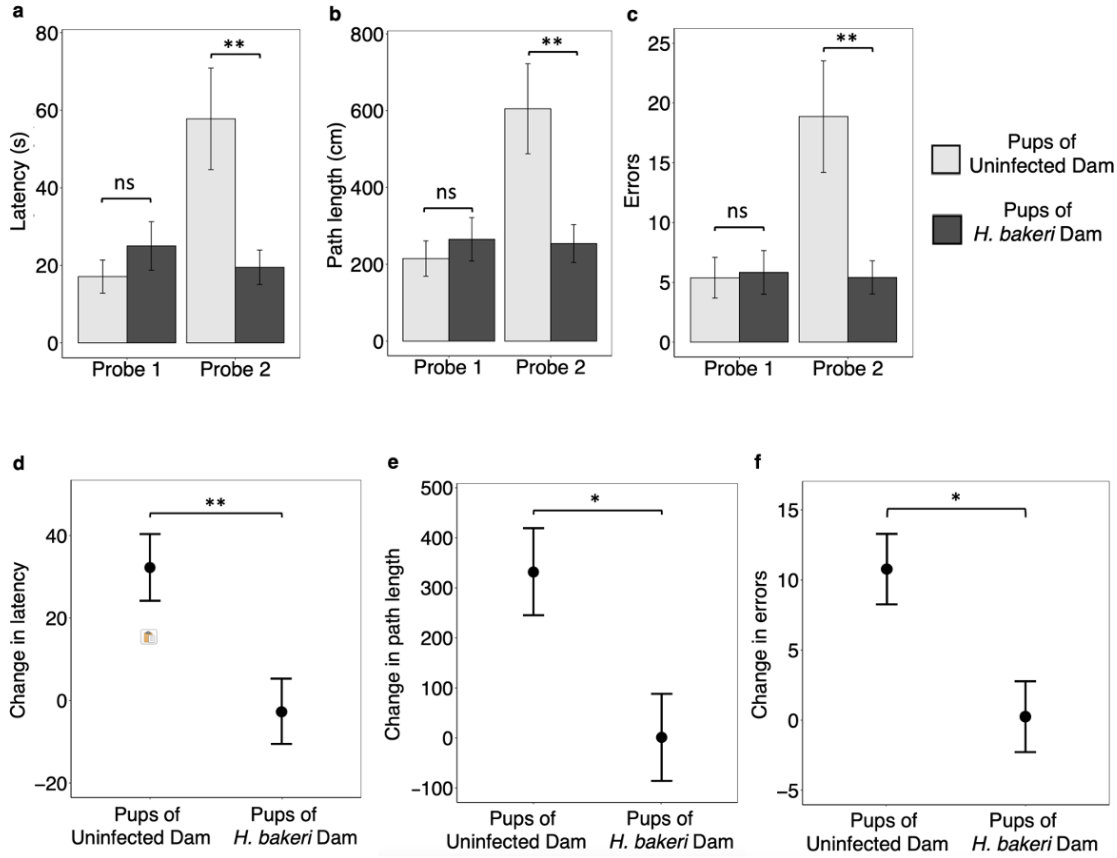


Figure 5. Schematic representing experimental design and protocol. Of the 19 timed-pregnant dams received on gestation day (GD) 4, only 16 delivered litters. On postnatal day (PD) 15, four pups per sex per litter were selected to perform behaviour tests, with half performing the Open Field Test and Object Location Test, and the other half performing the Barnes Maze Test. Of the pups selected for behavioural analysis, their size, specifically crown-rump length and weight, were recorded on PD 15 and 21.

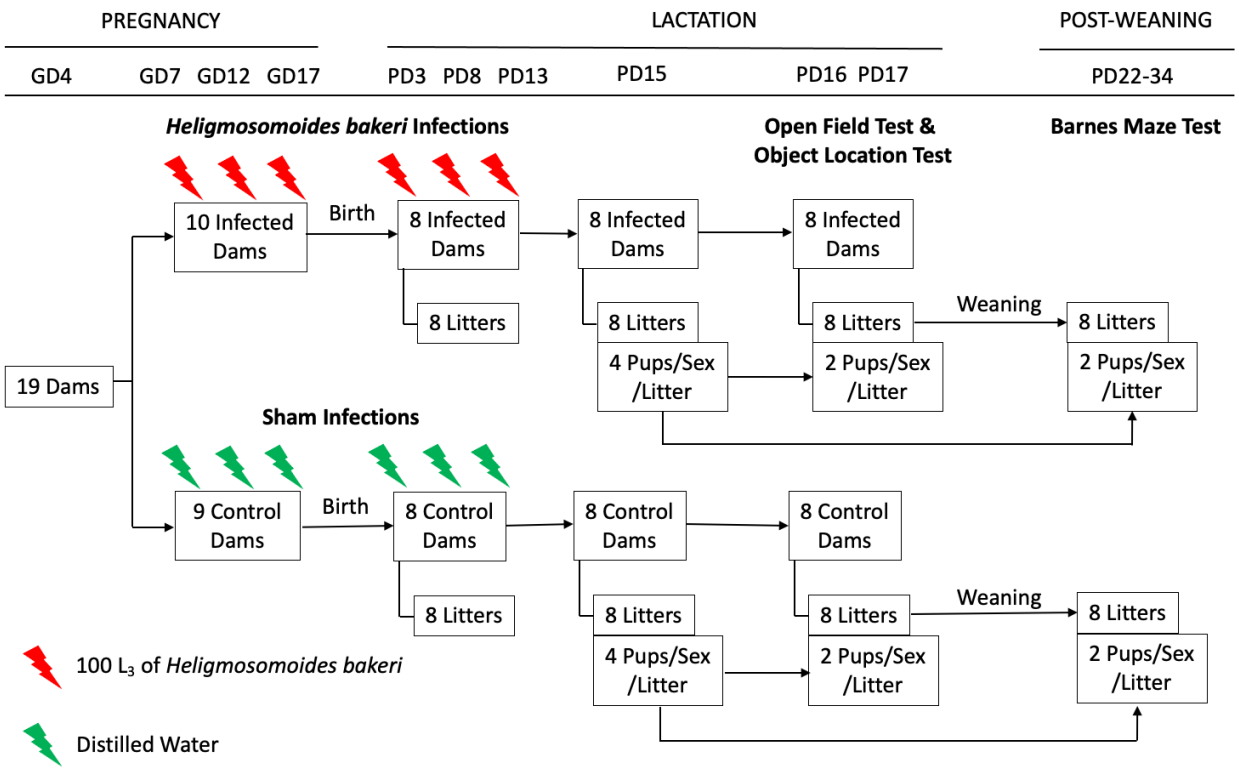
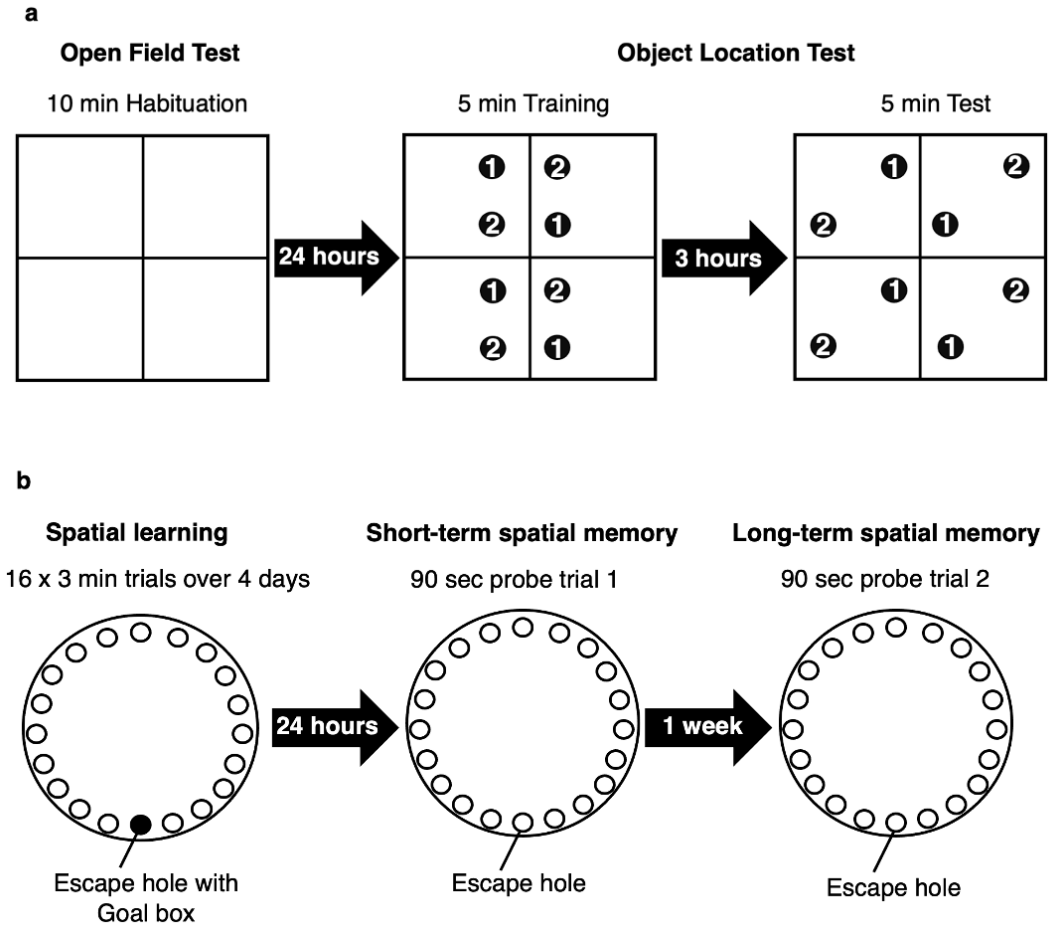
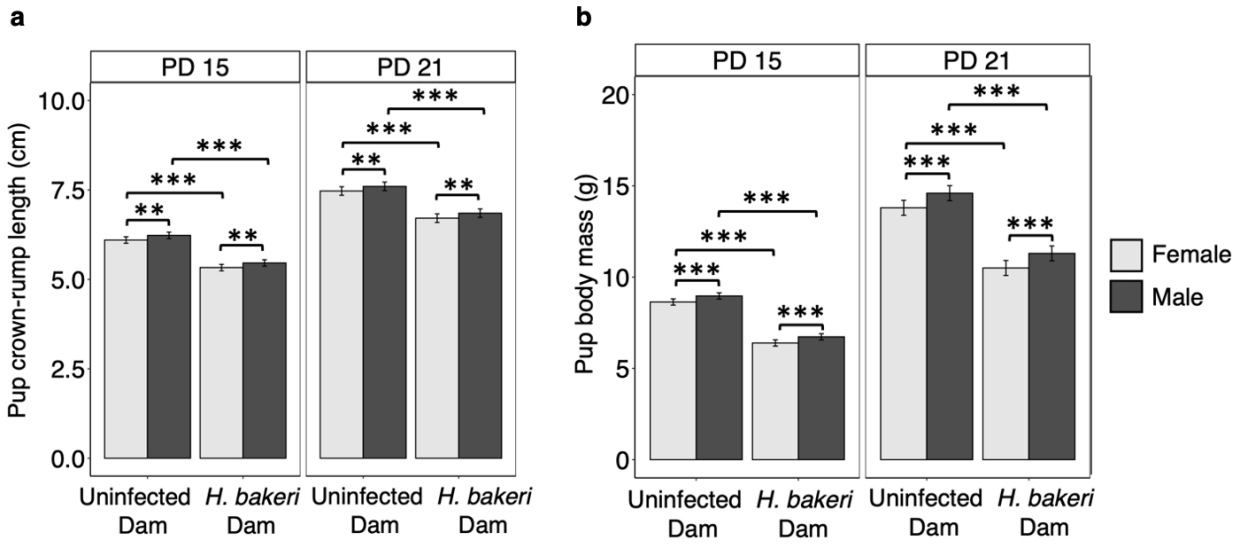


Figure 6. Bird's eye view of the experimental apparatus and protocols for the Open Field Test and Object Location Test, and the Barnes Maze Test. (a) Arena used for both Open Field Test and Object Location Test. Subjects were assigned to one of four identical plexiglass compartments. Two identical objects (Object 1, stationary object and Object 2, mobile object) were added to each compartment after the Open Field Test for the object location training, and the mobile object was moved to a novel location prior to the test trial. Subjects that recognize that Object 2 was in a different location are expected to increase their investigation of this object during the test trial. (b) Barnes Maze with 20 equally spaced holes, one of which is the escape hole with a goal box beneath it during the training but not the two probe trials. During training, subjects learn the location of the escape hole relative to spatial cues which surround the maze. Short term spatial reference memory was assessed in probe trial 1, one day after training, and long-term spatial reference memory was assessed during probe trial 2, one week later.



Supplementary Figures

Supplementary Figure 1. Effect of maternal *H. bakeri* infection and offspring sex on offspring size at postnatal day (PD) 15 and 21. Pups were nested within dam, and litter size was included as a covariate. Values are LSmeans±SEM, n = 32 male and 32 female pups from 8 uninfected dams and n = 31 male and 33 female pups from 8 infected dams (**$p < 0.01$, ***$p < 0.001$). a) pup crown-rump length and b) pup body mass.



Connecting Statement I

In Chapter III, using a 2 x 2 factorial design, I examined the effect of a GI nematode infection during pregnancy and lactation on the spatial memory of uninfected male and female juvenile offspring. The results showed that maternal *Heligmosomoides bakeri* infection allowed 17 day-old offspring to retain object location memories for 3 hours in the Object Location Test and three week-old offspring to retain long-term spatial reference memories for 7 days in the Barnes Maze Test, in contrast to offspring of uninfected dams who could not retain these memories. Thus, my data showed for the first time an enhancement of spatial memory in response to maternal GI nematode infection regardless of pup sex. As rodents are not capable of retaining object location memories for 2+ hours until PD 24[1], nor can they retain long-term spatial reference memories for 7 days until PD 60[2], these findings suggested that the maturational processes needed to recall these spatial memories were accelerated in response to maternal *H. bakeri* infection.

A previous study from our lab reported that maternal *H. bakeri* infection up-regulated the expression of genes associated with LTP and Th2/Treg pathways, including genes for the potent immunoregulatory cytokines IL-4 and TGF-β, in the brain of uninfected seven-day old male neonates [3,10]. This indicated that the Th2/Treg immune response in the *H. bakeri* infected mother may be transmitted to their offspring and mimicked in their brain, which in turn may be positively associated with offspring neurodevelopment, given that TGF- β and IL-4 are known to have measurable downstream effects on LTP and memory[22-26].

Given that brain gene expression data from one-week old male neonates indicate that a maternal *H. bakeri* infection may promote LTP and induce a Th2/Treg biased neuroimmune environment, and given that spatial memory in rodents is widely regarded as dependent on hippocampal LTP[65,274] and regulated by Th2/Treg cytokines[22,23,27], I hypothesized that maternal *H. bakeri* infection may enhance offspring hippocampal LTP, possibly through transfer of a Th2/Treg immune phenotype from the infected dam that protects the offspring from direct infection and extends to their developing brain, which may underlie the enhanced spatial memory I observed in Chapter III. Therefore, in Chapter IV, I examine the consequences of

maternal *H. bakeri* infection on offspring hippocampal LTP, gene expression and neuroimmunity, and resistance to direct infection as as an index of peripheral immunity.

NOTE: References for Connecting statement I can be found in the "Master List of References for All Non-Manuscript Sections" located at the end of the document.

Chapter IV - Maternal gastrointestinal nematode infection alters hippocampal neuroimmunity, promotes synaptic plasticity, and improves resistance to direct infection in offspring

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Abstract

The developing brain is vulnerable to maternal bacterial and viral infections which induce strong inflammatory responses in the mother that are mimicked in the offspring brain, resulting in irreversible neurodevelopmental defects, and associated cognitive and behavioural impairments. In contrast, infection during pregnancy and lactation with the immunoregulatory murine intestinal nematode, *Heligmosomoides bakeri*, upregulates expression of genes associated with long-term potentiation (LTP) of synaptic networks in the brain of neonatal uninfected offspring, and enhances spatial memory in uninfected juvenile offspring. As the hippocampus is involved in spatial navigation and sensitive to immune events during development, here we assessed hippocampal gene expression, LTP, and neuroimmunity in 3-week-old uninfected offspring born to *H. bakeri* infected mothers. Further, as maternal immunity shapes the developing immune system, we assessed the impact of maternal *H. bakeri* infection on the ability of offspring to resist direct infection. In response to maternal infection, we found an enhanced propensity to induce LTP at Schaffer collateral synapses, consistent with RNA-seq data indicating accelerated development of glutamatergic synapses in uninfected offspring, relative to those from uninfected mothers. Hippocampal RNA-seq analysis of offspring of infected mothers revealed increased expression of genes associated with neurogenesis, gliogenesis, and myelination. Furthermore, maternal infection improved resistance to direct infection of *H. bakeri* in offspring, correlated with transfer of parasite-specific IgG1 to their serum. Hippocampal immunohistochemistry and gene expression suggest Th2/Treg biased neuroimmunity in offspring, recapitulating peripheral immunoregulation of *H. bakeri* infected mothers. These findings indicate maternal *H. bakeri* infection during pregnancy and lactation alters peripheral and neural immunity in uninfected offspring, in a manner that accelerates neural maturation to promote hippocampal LTP, and upregulates the expression of genes associated with neurogenesis, gliogenesis, and myelination.

Introduction

Prenatal exposure to viral and bacterial pathogens has been identified as a risk factor for neurodevelopmental disorders, including autism spectrum disorder (ASD) and schizophrenia[1,2]. Rodent studies have shown that prenatal exposure to these pathogens activates microglia- and astrocyte-mediated neuroinflammation which impairs neuron and oligodendrocyte development and survival, reduces myelination, disrupts synaptic plasticity, and results in irreversible neurodevelopmental defects, impaired cognitive function, and abnormal behaviors[3]. Administration of anti-inflammatory agents to infected mothers during pregnancy and lactation has been effective in dampening neuroinflammation in offspring and preventing the emergence of neurodevelopmental defects and associated behaviors[4,5].

Gastrointestinal (GI) helminths are ubiquitous in mammalian populations and survive in the host by releasing a variety of immunoregulatory factors that allow them to evade the immune system[6,7]. Via their immunoregulatory abilities, GI helminths dampen inflammatory and pathologic processes and prevent or ameliorate a number of hyper-immune diseases; including, allergy, autoimmune, and inflammation-associated neurological diseases[6-8]. It has been proposed that prenatal exposure to GI helminths alters offspring immunity[8-10], which may have life-long consequences for their brain function and behavior[8].

Our lab has focused on the influence of maternal GI nematode infection during pregnancy and lactation upon neurodevelopment and cognitive function in offspring, using the murine laboratory model *Heligmosomoides bakeri* (also known as *Heligmosomoides polygyrus)*, a strictly intestinal nematode with a direct lifecycle[11]. Maternal *H. bakeri* infection allowed 17 day-old offspring to retain object location memories for 3 hours and three week-old offspring to retain long-term spatial reference memories for 7 days, in contrast to offspring of uninfected dams who could not retain these memories[12]. As rodents are typically not capable of retaining object location memories for 2+ hours until postnatal day (PD) 24, nor can they retain long-term spatial reference memories for 7 days until PD 60[13,14], these findings suggested that the maturational processes needed to recall these spatial memories were accelerated in response to maternal *H. bakeri* infection[12]. We also reported that maternal *H. bakeri* infection increased the

expression of genes associated with long-term potentiation (LTP) in the brain of uninfected neonates[15], raising the possibility that this could underlie the enhanced spatial memory[12].

LTP of glutamatergic synapses is a form of activity-dependent synaptic plasticity and a leading candidate for the neural substrate underlying learning and memory[16,17]. Spatial memory in rodents is widely regarded as dependent on hippocampal synaptic plasticity[18,19]. Induction of LTP by high frequency stimulation of hippocampal Schaffer collateral (CA3-CA1) synapses requires concerted activation of amino-3-hydroxy-5-methyl-4-isoxazole- propionic acid (AMPA)- and N-methyl-D-aspartate (NMDA)-type glutamate receptors[16,17]. NMDARs act as pre- and postsynaptic coincidence detectors that gate LTP induction in response to repeated glutamate synapse activity. Initial AMPAR activity in response to glutamate release depolarizes the postsynaptic cell, facilitating NMDAR-dependent calcium influx upon subsequent glutamate release during high-frequency stimulation (HFS)[16,17]. Calcium influx triggers post-synaptic biochemical cascades, through activation of calcium/calmodulin-dependent protein kinase II (CaMKII), protein kinase C (PKC), and mitogen-activated protein kinases (MAPK), that results in the phosphorylation and insertion of additional AMPARs into the post-synaptic membrane, thus strengthening the synaptic response to future glutamate release[20-22]. LTP in the mouse hippocampal slice preparation is found in the second postnatal week[23]; however depending on strain, this can be delayed until 4- to 5-weeks[24]. Neuroinflammation inhibits LTP, whereas several immunoregulatory factors can promote LTP[25]; as *H. bakeri* induces an immunoregulatory response, we hypothesized that maternal *H. bakeri* infection will induce an immunoregulatory response in the offspring hippocampus, promoting hippocampal LTP to explain the earlier development of spatial memory in the offspring of infected dams[12].

Immunity against *H. bakeri* relies on a strong T helper type 2 (Th2) immune response involving CD4+ Th2 cells, elevated interleukin (IL)-4, IL-5, IL-9, IL-10, and IL-13 cytokine secretion, high serum levels of IgE and IgG1 antibodies, and activation of alternatively activated macrophages, eosinophilia, and mastocytosis in intestinal tissue[11]. Adult worms, however, induce an immunoregulatory response which aids their long-term survival and involves proliferation of Foxp3+ CD4+ regulatory T (Tregs) cells, tolerogenic dendritic cells, and the potent immunoregulatory cytokines IL-10 and transforming growth factor-β (TGF-β)[11]. Maternal transfer of immunity both *in utero* and via nursing shapes the developing immune

system[10]. Consistent with this, maternal infection with GI nematodes can result in the transfer (via nursing) of maternally derived parasite-specific antibodies[26] and cells[9], which alters offspring immunity and protects them from direct infection. We reported that immune stimuli from the *H. bakeri* infected mother may also reach the brain of uninfected offspring, as Th2/Treg pathways were up-regulated in neonatal brains[15,27]. If the neuroimmune system is altered by maternal infection, glial cells (microglia, astrocytes and oligodendrocytes), which have vital roles in brain development and function, and are particularly sensitive to immune stimuli, are likely to be altered.

Microglia regulate neurogenesis, neuronal survival, and participate in synaptic pruning and maturation and are the resident immune cells of the central nervous system (CNS)[28,29]. They are highly plastic and take on a wide repertoire of states and functions depending on immune stimuli. In response to Th1/Th17 cytokines, microglia typically release inflammatory mediators, which if prolonged, can drive neuroinflammation and neurotoxicity[30]. Conversely, in response to Th2/Treg cytokines, microglia are associated with immune regulation, neuroprotection and tissue repair[30,31]. Astrocytes also respond to immune stimuli and are involved in the maintenance and regulation of neuronal function, synaptogenesis, neurotransmitter cycling, metabolic support of neurons, modulation of synaptic transmission and maintenance of the blood–brain barrier[29]. Oligodendrocytes generate and maintain myelin sheaths around axons, necessary for the rapid saltatory propagation of action potentials[32], with developmental myelination peaking during postnatal week three in mice[33]. Due to the vital roles of glia during neurodevelopment, alterations to their development or function in response to immune stimuli transferred from the *H. bakeri* infected mother may provide a mechanism underlying the behavioural changes previously observed[12].

We aimed to elucidate the mechanisms responsible for the enhanced spatial memory in the uninfected offspring of *H. bakeri* infected mothers[12]. Specifically, in response to this maternal infection, we found changes to LTP and gene expression in the uninfected offspring hippocampus that are consistent with improved performance in spatial memory. Coincident with these changes, we identified increased density of hippocampal microglia and astrocytes, a higher percentage of CD206 positive microglia, and increased expression of the TGF- β signaling pathway. We provide evidence that maternal GI nematode infection improves the resistance of

juvenile offspring to direct infection, shifts the peripheral and neural immune response toward a Th2/Treg phenotype, promotes development of hippocampal LTP and upregulates genetic markers of neurogenesis, gliogenesis and myelination, all consistent with earlier development of spatial memory.

Results

Here we assessed the influence of maternal *H. bakeri* infection on hippocampal gene expression, the capacity to induce LTP, the neuroimmune system, and resistance to direct infection in offspring. Outbred CD-1 mice were repeatedly infected (or sham-infected) during pregnancy and lactation. Juvenile offspring in litters from 18 uninfected and 20 *H. bakeri* infected dams were used. Mortality was consistently zero in this infection model, as expected[12].

Maternal *H. bakeri* infection did not influence dam weight or litter size but lowered offspring weight and length.

H. bakeri did not significantly affect maternal body weight at gestation day (GD 7), 12 or 17 (all P values > 0.9, Supplementary Fig. 1) or litter size (uninfected: 12.6 ± 0.3 vs. infected: 11.8 ± 0.4; t = 1.66, df = 36, p = 0.11). As is typical of a maternal *H. bakeri* infection[12], pups born to infected dams had shorter length and lower mass than pups of uninfected dams at PD 20 (all P values < 0.0001, Supplementary Fig. 2).

Maternal *H. bakeri* infection altered hippocampal gene expression in uninfected offspring.

To investigate the mechanisms that underlie the previously reported enhancement of spatial memory[12], we assessed changes in the gene expression profile in the hippocampus of PD 23 pups in response to maternal *H. bakeri* infection. After filtering and normalization of high throughput RNA-seq data, we identified 16,143 genes for differential gene expression analysis. Principal component analysis (PCA) showed no pattern with respect to sex, and differential expression analysis (DEA) of males *vs*. females yielded only 10 differentially expressed genes (DEGs). Offspring sex was therefore excluded as a variable from the analysis.

With respect to maternal infection, PCA showed DEGs in two clear clusters, based on treatment, indicating a strong influence of maternal infection on offspring hippocampal gene expression (Figure 1a). DEA identified 1,753 up-regulated, and 1,550 down-regulated genes in the pups of *H. bakeri* infected dams (Figure 1b, Supplementary Table 1). Hypergeometric tests identified 38 KEGG pathways and 67 GO BP terms that were overrepresented in the list of DEGs (FDR < 0.05; Supplementary Table 2). Of particular interest, the LTP (FDR = 0.02; Figure 2a), glutamatergic synapse (FDR = 0.001; Supplementary Table 3), MAPK signaling (FDR = 0.009; Supplementary Table 4), neurogenesis (FDR = 1.26E-05; Supplementary Table 5), gliogenesis (FDR = 0.0001; Supplementary Table 6), and TGF-β signalling (FDR = 0.03; Figure 2b) pathways had higher expression levels in offspring from infected mothers. A number of markers associated with microglia (*Hexb*, *Sall1*, *Tgfbr1, Mef2a, Golm1, Tmsb4x,* and *Tppp*), astrocytes (*NFIA*, *NFIB, GFAP*, *S100B* and *Aqp4*) and oligodendrocytes (*Olig1*, *Olig2*, *Sox10*, *Nkx2.2, Myrf, Zfp488, Cldn11*, *Plp1, Foxo4* , *Cnp*, *Mbp, Mag and Mog*) were also up-regulated in response to maternal infection (Figure 3, Supplementary Table 1).

Maternal *H. bakeri* infection enhanced hippocampal LTP in uninfected offspring.

In a mouse strain similar to the one we used here, a progressive developmental increase in the capacity to induce hippocampal CA1 LTP has been documented. LTP lasting >60 min was found in 0% of slices from 2 week-old mice, 26% of slices from 3-4 week-old mice, and 69% of slices from 5 week-old mice[24]. To determine whether maternal *H. bakeri* infection accelerates the development of hippocampal LTP, we used acute hippocampal slices from 21-24 day old male pups to record field excitatory postsynaptic potentials (fEPSPs) in the CA1 stratum radiatum, evoked by CA3 Schaffer collateral stimulation.

Stimulus input/output (I/O) plots showed no difference in fEPSP slope over increasing stimulation intensity between groups ($F_{1,12} = 0.21$, $P = 0.66$; Fig. 4a), indicating that basal synaptic transmission was generally unaltered by maternal infection. Paired-pulse facilitation ratio (PPF), a proxy measure of presynaptic release probability[34], also showed no significant difference between groups across inter-pulse intervals ($F_{1,12} = 2.53$, $p = 0.14$; Fig. 4b), suggesting presynaptic release was also generally unaltered in pups by maternal infection.

LTP was induced by HFS only in pups of infected dams (Fig. 4c, d). In pups of infected dams, the fEPSP slope measured 60 min after the last HFS was 124% ± 7.1% of baseline, a value significantly higher than the fEPSP slope in pups of uninfected dams (84.7% ± 15.9% of baseline) ($t = 2.23$, $df = 12$, $p=0.04$), indicating an enhanced capacity to induce LTP in response to maternal infection. Also, a higher proportion of slices from pups of infected mothers maintained LTP for >60 mins (5/7 = 71%), compared to pups of uninfected mothers (1/7 = 14%). The data suggest the capacity to induce and maintain LTP is accelerated as a result of maternal *H. bakeri* infection.

Maternal transfer of *H. bakeri*-specific IgG1 and increased resistance to infection.

As the maternal immune system influences that of their offspring, and the immune system can strongly influence neurodevelopment and behaviour, the impact of maternal infection on the ability of juvenile male and female offspring to resist a direct *H. bakeri* infection was assessed as an index of peripheral immunity. Serum from PD 24 uninfected offspring of *H. bakeri* infected dams had readily detectable levels of *H. bakeri*-specific IgG1 antibody, whereas pups of uninfected dams did not (Figure 5a). At PD 27, offspring of *H. bakeri* infected and uninfected dams were experimentally infected with 150 *H. bakeri* larvae. One-month later, infection intensity was significantly lower in pups of *H. bakeri* dams compared to pups of uninfected dams, as indicated by fewer eggs per gram of faeces ($p = 0.003$; Figure 5b), worm burden ($p = 0.063$; Figure 5c), and fecundity ($p = 0.005$; Figure 5d). No effect of pup sex was detected (all P values > 0.17, data not shown).

Maternal *H. bakeri* infection increased glial density in hippocampus of uninfected offspring.

To determine if maternal *H. bakeri* infection altered the neuroimmune system in the hippocampus of PD 22 uninfected offspring, brain sections were immunohistochemically labelled for astrocytes and microglia with antibodies against GFAP and Iba1 respectively. To gain insight into the functional role of microglia, we also immunostained for CD206, a microglial cell surface protein typically increased in response to Th2 cytokines[30,31,35]. Cell

density was assessed in the dorsal hippocampus, with a focus on the CA3-CA1 region (Supplementary Fig. 3). Pups of *H. bakeri* infected dams had higher density of cells positive for GFAP ($F_{1,20} = 6.22$, $p = 0.02$; Fig 6a, c) and Iba1 ($F_{1,20} = 11.81$, $p = 0.003$; Fig 6b, d), and a higher percentage of Iba1+/CD206+ cells ($F_{1,20} = 5.09$, $p = 0.04$; Fig 7a, b), relative to pups of uninfected dams. Pup sex did not affect astrocyte or microglia density, nor the percentage of Iba1/CD206 double positive cells ($p > 0.48$, data not shown). Together with gene expression data, the results suggest maternal *H. bakeri* infection promotes a Th2/Treg biased immune response in the hippocampus of uninfected offspring.

Discussion

Here we used a mouse model to examine the influence of a maternal GI nematode infection on hippocampal gene expression, LTP and the neuroimmune system, as well as resistance to direct infection of three week-old juvenile offspring. We observed four key consequences of maternal infection: 1) earlier onset of the capacity to induce hippocampal LTP, with changes in gene expression suggesting this may be due to accelerated maturation of glutamatergic synapses; 2) higher levels of hippocampal gene expression in neurogenesis and gliogenesis pathways, and higher levels of gene expression associated with oligodendrocytes and myelination; 3) greater resistance to *H. bakeri* infection, evidenced by lower worm burden and parasite fecundity, consistent with maternal transfer of parasite-specific IgG1 to the serum of uninfected offspring; and 4) an increase in the density of hippocampal microglia and astrocytes, a higher percentage of CD206 positive microglia, and increased expression of the TGF- β signaling pathway involved in immune regulation. Together, the data suggest immune stimuli from the *H. bakeri* infected mother are transferred to the uninfected offspring, extend to their brain, and result in a Th2/Treg-biased neuroimmune response that underlies accelerated hippocampal maturation as evidenced by enhanced LTP, increased expression of genes associated with neurogenesis, gliogenesis, and myelination, and enhanced spatial memory [12].

Maternal *H. bakeri* infection enhanced or accelerated the developmental capacity for LTP induction in uninfected offspring, which may be due to the observed changes in gene expression. As the capacity for hippocampal LTP is believed to strongly correlate with successful spatial

memory formation [19], this is consistent with the enhanced spatial memory we previously detected in offspring of *H. bakeri* infected dams[12]. To our knowledge, this has not been described in other maternal infection models, although similarly to our observations here, exposure to exercise during early life enhances LTP and spatial memory[36]. In contrast, maternal exposure to *Escherichia coli* lipopolysaccharides[5,37] or stress[38], both of which are known to induce neuroinflammation in offspring[3,39], impairs hippocampal LTP and spatial memory in offspring.

Consistent with altered LTP, we found increased expression of genes associated with glutamatergic synapses and the MAPK signalling pathways associated with LTP, in addition to those associated with AMPAR and NMDAR subunits, CAMKII and PKC, each of which may contribute to LTP induction and maintenance. GluR1 AMPAR subunits are critical for LTP and memory retention[40]. Exposure of pregnant mice to a bacterial mimic reduced GluR1 in offspring hippocampi and impaired spatial memory[41], whereas increased hippocampal GluR1 improved spatial memory in rats[42,43]. Hippocampal LTP also relies on NR2A-containing NMDARs[44]. Exposure of pregnant mice to a viral mimic decreased NR2A in offspring hippocampi and impaired spatial memory[45]. In contrast to inflammatory maternal infections, maternal *H. bakeri* infection resulted in increased expression of the GluR1 and NR2A genes, *Gria1 and Grin2a,* whereas expression of the GluR4 gene, *Gria4,* was reduced. As *Gria1* and *Grin2a* subunit expression increases during normal neurodevelopment, and *Gria4* decreases[46-48], our results suggest that maternal *H. bakeri* infection accelerates hippocampal glutamate synapse maturation.

CaMKII and PKC are calcium-dependent-kinases with important roles in LTP; both are linked to positive regulation of GluR1 AMPAR subunit phosphorylation, which increases AMPAR conductance and postsynaptic responsiveness[20-22]. Injection or overexpression of CAMKII [49-51] or PKC [52,53] enhances LTP and spatial memory. CaMKII has four distinct isoforms (α, β, γ, and δ)[54], and PKC consists of at least 10 isoforms, including the classical subfamily of PKC isozymes (PKCα, PKCβ and PKCγ) [55]. In response to maternal *H. bakeri* infection, we detected increased expression of CaMKII α and β genes (*Camk2a* and *Camk2b,* respectively), and the PKCα gene, *Prkca*. Loss of CaMKIIα, CaMKIIβ[56-59] or PKCα[55] results in severe impairment of LTP and spatial memory. Taken together, higher expression of *Gria1*

and *Grin2a,* lower expression of *Gria4,* with higher expression of *Camk2a*, *Camk2b* and *Prkca* would be expected to promote LTP, as demonstrated here, and facilitate spatial memory in response to maternal infection, as previously reported[12].

The increased gene expression associated with neurogenesis pathways may also contribute to enhanced spatial memory in juvenile offspring[12]. In the adult brain, neurogenesis in the hippocampal dentate gyrus contributes to spatial memory[60], and maternal physical exercise increases offspring hippocampal neurogenesis and spatial memory, via brain derived neurotrophic factor (BDNF)[61-64]. BDNF is a key positive modulator of LTP and neurogenesis[65]. Here we found increased hippocampal *Bdnf* expression in response to maternal infection ($p = 0.015$, adjusted p value = 0.067). Further studies are required to determine how enhanced hippocampal neurogenesis may contribute to the enhanced spatial memory detected in juvenile offspring of *H. bakeri* infected dams[12].

Our findings support the hypothesis that maternal GI nematode infection alters hippocampal function and spatial memory in developing uninfected offspring by transfer of maternal immunity, as offspring immunity strongly influences neurodevelopment and behaviour[8]. Maternal immune antibodies and cells are transferred to young offspring via nursing, offering protection from pathogens and shaping immune system maturation[10]. For example, via nursing, *H. bakeri*-specific IgG1 antibody is transferred from infected mothers to pre-weaned 10-day old neonates, providing protection against *H. bakeri* [26], and maternally derived Th2 CD4+ T cells are transferred from *Nippostrongylus brasiliensis* infected mothers to offspring, providing long-lasting cellular immunity against direct infection with this nematode[9]. Our hypothesis that the Th2/Treg immune response in the *H. bakeri* infected dam is mimicked in 3-week-old uninfected weaned offspring is supported by *H. bakeri*-specific IgG1 in the serum of the uninfected offspring of infected dams. Further, fewer adult worms with reduced fecundity in *H. bakeri* infected offspring of infected dams, compared with infected offspring of uninfected dams, indicates heightened resistance and that the functional immunity induced by maternal infection parallels that seen during a secondary challenge in this mouse model[11].

Additionally, *H. bakeri*-specific antibodies in offspring serum parallel a Th2/Treg biased neuroimmune response in the hippocampus, which may alter glial differentiation, development

and function. This may support LTP, neurogenesis, gliogenesis, and myelination, and contribute to improved spatial memory[12]. We previously reported that whole brain samples of uninfected seven-day old male neonates of *H. bakeri* infected dams had up-regulated expression of IL4 and TGF-β genes[15,27]. These cytokines are hallmarks of *H. bakeri* infection[11], and vital for the regulation of brain immunity with downstream effects on LTP, neurogenesis, and spatial memory[66-71]. Consistent with this, we detected higher expression of the TGF-β signaling pathway and of *Tgfb2* and *Tgfbr1* genes but downregulation of the *Tgfb3* gene in the hippocampus of uninfected juvenile offspring. Of the three isoforms of TGF-β, encoded by the genes *Tgfb1-3*, TGF-β2 regulates hippocampal synaptic plasticity[68] and neurogenesis[69], and TGF-β2 knockout mice exhibit synaptic and cognitive dysfunction[72,73]. Further, gene expression and protein levels of TGF-β2 and its receptor, TGF-β receptor 1 (TGF-βR1), are higher in IL-4 induced microglia[74], which are associated with immune regulation and memory[75]. In contrast, TGF-β3 promotes Th17 cell differentiation and the pathogenesis of autoimmune diseases[76,77]. The up-regulated expression of both *Tgfb2* and *Tgfbr1* genes, and downregulation of the *Tgfb3* gene suggests a TGF-β immunoregulatory response in the hippocampus of the uninfected offspring.

TGF-β is also critical for differentiation, development and function of neurons and glia[69,78]. The higher density of microglia and higher expression levels of microglia-specific genes (*Hexb*, *Sall1*, *Tgfbr1, Mef2a, Golm1, Tmsb4x,* and *Tppp*), higher density of astrocytes and higher expression levels of astrocyte-specific genes (*Nfia*, *Nfib, Gfap*, *S100B* and *Aqp4*), and greater percentage of CD206 positive microglia in the hippocampus of offspring of *H. bakeri*-infected dams indicate a hippocampus responding to Th2/Treg factors. CD206, like TGF-β2 and TGF-βR1, is typically increased by microglia responding to IL-4, suggesting a microglia phenotype associated with immune regulation[30,31,35,74]. Expression of *Tgfbr1, Hexb, Golm1*, and *Sall1* increase with microglia maturity, and maturation is dependent on TGF-β signaling [78,79] which was up-regulated by maternal infection. *Nfia* and *Nfib* contribute to astrocyte development, *Gfap*, *S100B* and *Aqp4* are markers of mature astrocytes[80], and *Aqp4* is important in TGF-β-associated immunoregulation[81].

TGF-β signaling is also critical for oligodendrogenesis and developmental myelination[70]. In response to maternal *H. bakeri* infection, we detected higher expression of 13

key genes associated with oligodendrocytes and myelination. *Olig1*, *Olig2*, *Sox10*, *Nkx2.2, Myrf, Zfp488* and *Cldn11*, are necessary for oligodendrocyte differentiation and myelination during development[32,82]. *Plp1, Foxo4* and *Cnp* are expressed during oligodendrocyte differentiation, and mature myelinating oligodendrocytes express *Mbp, Mag and Mog*[32]. Delayed hippocampal myelination leads to impaired excitatory synaptic transmission and cognitive dysfunction[4,83,84], whereas early developmental hippocampal myelination promotes excitatory synaptic transmission and cognitive function, including spatial memory[85]. Thus, accelerated oligodendroglial maturation and myelination in the developing hippocampus via TGF-β signaling could further contribute to enhanced LTP and spatial memory[12] observed in juvenile offspring in response to maternal *H. bakeri* infection.

It is worth highlighting the contrasting consequences of maternal bacterial and viral infections with intestinal nematode infections. Maternal bacterial or viral infections induce a strong Th1/Th17 immune response in the mother that extends to the offspring brain, and is associated with microglia- and astrocyte-mediated neuroinflammation. If prolonged, neuroinflammation drives oligodendroglial apoptosis, delays myelination, and impairs LTP and neurogenesis, ultimately resulting in the emergence of ASD-like behaviours and cognitive impairments in the offspring[3-5,83,84,86]. Conversely, our data demonstrate that maternal intestinal nematode infection induced a Th2/Treg immunoregulatory environment in the developing hippocampus, that was associated with upregulated genetic markers of neurogenesis, gliogenesis and myelination, and enhanced LTP and spatial memory[12]. We hypothesize that these differences result from the transfer of Th2/Treg-specific immune molecules from the nematode-infected mother but do not exclude other pathogen-related differences in the maternal microbiome that may affect microbial colonization of the offspring[87,88]. Regardless, if the observed changes induced by maternal nematode infection persist as pups grow, and if damaging neurological changes do not occur, this raises the possibility that GI nematodes might be important for proper brain development and function, and may provide a promising avenue for preventing inflammation-associated neurodevelopmental disorders.

We acknowledge the following limitations. Our electrophysiology experiments recorded LTP in male but not female offspring. We hypothesize that similar results would have been seen in females as sex did not affect spatial memory of juvenile offspring of *H. bakeri* infected

mothers[12] or hippocampal gene expression or microglia/astrocyte density. Also, data from only the first slice tested per animal was used in our LTP experiment as subsequent slices were of lower quality, limiting our sample size, however, this avoided pseudoreplication. We also acknowledge that new hypotheses are based on gene expression data. Thus, it will be important to confirm our gene expression findings by assessing protein levels. For instance, it will be of great interest to perform unbiased stereology on BrdU/NeuN-double-labeled cells in the dentate gyrus to determine if maternal *H. bakeri* infection enhances hippocampal neurogenesis, as well as to assess cell density of oligodendrocytes and myelination, and levels of key cytokines (IL-4 and TGF-β) in this model. Finally, caution is needed in interpreting the function of microglia and astrocytes given their high plasticity.

To the best of our knowledge, this is the first study to show that a maternal GI nematode infection promotes hippocampal LTP and upregulates genetic markers associated with neurogenesis, gliogenesis and myelination in the uninfected juvenile offspring, possibly through transfer of a Th2/Treg immune phenotype from the infected dam that protects the offspring from direct infection and extends to their developing brain. These findings identify possible mechanisms underlying our previous observation of enhanced spatial memory in two and three week-old offspring exposed to *H. bakeri* maternal infection[12]. These positive effects on neurodevelopment and cognition identify a potential unappreciated benefit of maternal GI nematode infection. Given the immunoregulatory nature of this parasite, that extends to the offspring, our findings may be valuable in efforts to prevent the development of inflammation-associated neurodevelopmental disorders, like ASD.

Methodology

Experimental design.

We compared juvenile offspring of *H. bakeri* infected versus uninfected dams.

Mice and Parasites.

38 primiparous eight week-old timed pregnant (GD 4) outbred CD-1 mice were received at McGill Macdonald Campus' Animal Facility from Charles River Laboratories, Quebec,

Canada. Each dam with her litter was housed individually in a Nalgene cage (Fisher Scientific, Canada) at 21–23 °C, 40–60% relative humidity and a 12 h light and dark cycle. Mice had ad libitum access to a 2920X Teklad rodent diet (18% crude protein, 5% crude fat, 5% crude fiber). Within each of the eight staggered groups of dams received, dams were randomized into uninfected and *H. bakeri* infected groups, with a total of 18-20 dams per group. Using standard *H. bakeri* protocols[89], infective third-stage larvae (L3) were obtained by fecal culture of stock parasites maintained in outbred CD-1 mice. Dams in the *H. bakeri* group were infected using an oral gavage needle with 100 ± 3 L3 suspended in 0.1 mL distilled water on GD 7, 12, 17, and PD 3, 8 and 13. Uninfected dams received 0.1 mL distilled water via oral gavage at the same frequency. Given that *H. bakeri* eggs released into the environment develop into L3 after 7 days, all cages were cleaned every 5 days to ensure offspring could not ingest L3. Dams were weighed on GD 7, 12 and 17 to ensure infection did not result in weight loss. Following weaning (PD 20), dams were euthanized and necropsied and successful infection of dams was confirmed by noting presence of adult worms in the small intestine.

Pups were born on GD 19 and litter size was recorded. At PD 20, pups were weaned, sexed, given a unique identifier, and body mass and length from the top of the head to the base of the tail recorded. On PD 21, a subset of pups were transported to the Montreal Neurological Institute's Animal Facility for the experiments outlined below. Pups within each litter were randomly selected for each experiment. At euthanasia, experimental pups were necropsied and intestines were examined for adult *H. bakeri*. This confirmed that the offspring had not been accidentally infected. Pups not used for this study were assigned to a separate study.

Compliance with guidelines for research with experimental animals.

This study (protocol #2000– 4601) was approved by the McGill University Animal Care Committee according to the guidelines of the Canadian Council on Animal Care. All methods were carried out in accordance with relevant guidelines and regulations, and the study was carried out in compliance with ARRIVE guidelines (https://arriveguidelines.org).

Gene expression study.

Tissue samples. On PD 23, three male and six female offspring from uninfected dams and five male and five female offspring from *H. bakeri* dams (no more than one pup/sex/dam) were decapitated without anesthesia, as anesthesia can influence gene expression in the brain. The brain was rapidly removed and using iced cold artificial cerebrospinal fluid (aCSF), hippocampi were rapidly removed bilaterally, immediately flash frozen in liquid nitrogen, then stored at -80°C. Hippocampal samples were sent to Genome Quebec for total RNA extraction and sequencing and FASTQ files were obtained. No pooled samples were used.

Homogenization. 900µL of RNeasy Plus Universal Mini Kit provided lysis buffer reagent (i.e. QIAzol) was added to previously weighted tissue (10-15 mg). Homogenization was done using a QIAGEN TissueLyser II with 5 mm stainless beads, for 2 cycles of 30Hz x 2 min plus 1 cycle of 30 Hz x 1 min.

Extraction. Total RNA extraction was performed using the RNeasy Plus Universal mini kit (QIAGEN, cat.73404) according to the manufacturer's instructions. RNA was eluted in 35µl buffer provided with the extraction kit. RNA quality was determined by the RNA Integrity Number (RIN), measured by 2100 Bioanalyzer (Agilent Technologies) using RNA 6000 Nano kit, following the manufacturer's protocol.

Library preparation. Libraries were generated from 250 ng of total RNA using the Illumina® Stranded mRNA Prep, Ligation Kit (Illumina), as per the manufacturer's recommendations. Libraries were quantified using the KAPA Library Quanitification Kits - Complete kit (Universal) (Kapa Biosystems). Average size fragment was determined using a LabChip GXII (PerkinElmer) instrument.

Sequencing. Libraries were normalized and pooled and then denatured in 0.02N NaOH and neutralized using HT1 buffer. The pool was loaded at 175pM on an Illumina NovaSeq 6000 S4 lane using Xp protocol as per the manufacturer's recommendations. The run was performed for 2x100 cycles (paired-end mode). A phiX library was used as a control and mixed with libraries at 1% level. Base calling was performed with RTA v3. Program bcl2fastq2 v2.20 was then used to demultiplex samples and generate FASTQ reads.

Gene expression analysis. Raw reads were aligned to the mouse GRCm38 reference transcriptome using the Kallisto software[90] (version 0.46.1, minimum quality score = 25). Transcripts were filtered to remove those with low abundance and low variability across all samples (abundance < 4 counts, removed 15th percentile with lowest variability). Counts were normalized and converted into log2-counts-per-million (logCPM) using the Relative log expression normalization method as implemented in the edgeR R package[91] (version 3.38.4). Differential expression analysis (DEA) of the logCPM values was conducted with the edgeR R package to identify genes with significantly different expression between offspring of *H. bakeri*-infected or uninfected mothers (adjusted p-value < 0.05, FDR method). Since principal component analysis showed no pattern with respect to sex in the top components and DEA of male versus female yielded only 10 differentially expressed genes (DEGs), offspring sex was excluded from the analysis. Hypergeometric tests were used to identify gene sets (KEGG and GO BP) that were significantly overrepresented in the list of DEGs (adjusted p-value < 0.05, FDR method). Analyses were performed twice, once using the list of DEGs with positive log2FC, and once using the list with negative log2FC, to identify overrepresented pathways in the list of up-regulated and down-regulated DEGs. All gene expression analysis was conducted using the ExpressAnalyst software (https://www.expressanalyst.ca/), a web-based platform for processing, analyzing, and interpreting RNA-sequencing data[92].

Long-term potentiation study.

Brain slice preparation. On PD 21-24, seven male offspring from *H. bakeri* infected or uninfected dams (no more than one pup/dam) were decapitated without anesthesia and the brain was immediately removed and submerged in ice-cold oxygenated (95% O_2/5% CO_2) artificial cerebrospinal fluid (aCSF) (in mM: 120 NaCl, 3 KCl, 2 $MgSO_4$, 2 $CaCl_2$, 1.2 NaH_2PO_4, 23 $NaHCO_3$, 11 glucose) for one min. The brain was placed on an iced-cold platform with aCSF and both hippocampi were rapidly removed. Transverse hippocampal slices (400 µm) were cut with a tissue chopper (MclLwain, TC752). Approximately three slices from the middle third of the hippocampus were obtained from each hemisphere. Slices were kept in chilled and oxygenated aCSF and the hippocampal CA3 region was removed with a scalpel. Slices were then placed in a humidified and oxygenated (95% O_2/5% CO_2) interface chamber (Digitimer, BSC2-2) perfused

(0.15 ml/min) with aCSF at 28-30°C. Slices were left to recover for at least 1.5 hr before recording.

Electrophysiology. Extracellular recording pipets (1.5–3 MΩ) encasing a chlorinated silver wire stripped at the tip were pulled from borosilicate glass capillary tubing (Warner Instrument, Hamden, CT), filled with 4 M NaCl and placed in stratum radiatum of CA1 to record field excitatory postsynaptic potentials (fEPSPs). Synaptic events were evoked by Schaffer collateral stimulation by placing a concentric bipolar stimulating electrode (FHC Inc., Bowdoin, ME) (~500 um lateral from the recording electrode) in stratum radiatum of area CA1.

Slices were stimulated every 10 sec and an input-output (I/O) curve generated by measuring the slope (mV/ms) of the extracellular field excitatory postsynaptic potentials (fEPSPs) in response to increasing stimulus intensities (ranging from approx. 0-400 μA). Stimulus intensity was increased to the point where a population spike was just detectable in the fEPSP record, and the test response was then set at 50% of this stimulus intensity. Paired-pulse facilitation ratio (PPF) was then assessed as an increase in the size of the synaptic response to a second pulse delivered within a short interval of time following the first pulse. PPF is a form of short-term synaptic plasticity that results primarily from presynaptic mechanisms and is generally explained as an increase in the probability of vesicular release during the second stimulus, arising from prior accumulation of residual calcium[34]. PPF can be used to help determine if any differences observed in LTP are associated with presynaptic involvement[34]. To this aim, paired stimuli to the Schaffer collaterals were applied at increasing interpulse intervals (ranging from 20 - 220 ms at 40 ms increments) and the paired-pulse facilitation ratio was determined as the slope of the second fEPSP divided by that of the first fEPSP. Following PPF, baseline responses to stimulation at a frequency of 1 pulse every 20 sec were recorded. Once a stable baseline response had been established for 30 min, a high frequency stimulation (HFS) (3x100 Hz for 1 sec, with 10 sec between each 100 Hz train; repeated 3x at 5 min intervals) was applied, and responses were measured every 20 sec for 60 min after the HFS. Data were analyzed using Clampfit software (Version 10.7). Fiber volley amplitude (which is an indication of the presynaptic action potential arriving at the recording site) was measured during the I/O curve and throughout the LTP experiment to ensure it remained stable. The initial slope of the fEPSP was used as a measure of synaptic strength as this is preferred over the potential

amplitude to avoid contamination of the fEPSP by a population spike. The baseline response for the LTP experiments was calculated as the average response generated 5 min before HFS. All values were then converted to a percent change from the average baseline. A slice was considered potentiated if it remained ≥ 120% of baseline at 60 min[24]. Of note, there was only access to one interface recording chamber, preventing synchronous testing of multiple slices per mouse. Data from only the first slice tested per animal was used as subsequent slices were of lower quality, avoiding pseudoreplication.

Statistics. Data was analyzed in GraphPad Prism (Version 10.0.2) to compare offspring from *H. bakeri* infected dams with offspring from uninfected dams. Mixed models were used to test for differences in the I/O curve, PPF and LTP data between groups, followed by Sidak multiple comparisons test. Unpaired t-tests were conducted to compare the response between groups generated at 60 min following HFS. Values are presented as means ± SEM. The significance level was set at 0.05.

Resistance study.

Serum collection and ELISA for H. bakeri specific IgG1. On PD 24, eight offspring (4 per sex) from *H. bakeri* infected and four offspring (two per sex) from uninfected dams (no more than one pup/sex/dam) were anesthetized with isoflurane and blood samples were collected via cardiac puncture. Serum was stored at -20°C.

H. bakeri-specific IgG1 antibody absorbance curves were obtained via enzyme-linked immunosorbent assays (ELISA). *H. bakeri* excretory-secretory antigen (HES) was made using the well-established protocol by Stevenson et al. 2014[93]. An ELISA plate (Nunc Maxisorp) was coated with 50 μL HES diluted in PBS to 1μg/ml overnight at 4°C. The plate was washed 5 times with ELISA wash buffer (PBS + 0.05% Tween-20) and then blocked with 2X Ebioscience Assay Buffer A (PBS with 1% Tween-20 and 10% BSA) for 2 hours at room temperature (RT). The plate was then washed 5 times with ELISA wash buffer. 100 μL serum was added to each well using 5-fold serial dilutions, with a total of 8 serial dilutions (the starting dilution was 3-fold). Serial dilutions were done using 1X Ebio Assay Buffer A. Serum was incubated for 2 hr at RT. The plate was washed 5 times with ELISA wash buffer. Primary Antibody (Rat Anti-Mouse

IgG1-BIOTIN Clone SB77E: Southern Biotech #1144-08) was diluted 5000x in 1x Ebio Assay Buffer and 100 μl was added to each well and incubated for 1 hr at RT. The plate was washed 5 times with ELISA wash buffer. 100 μl of Secondary Antibody (Streptavidin-HRP: Southern Biotech #7100-05) was diluted 1000x in 1x Ebio Assay Buffer and 100 μl was added to each well and incubated for 1 hr at RT. Plate was washed 10 times with ELISA wash buffer. 100 μl of TMB substrate solution was added and the reaction was stopped by adding 100 μl 1M sulfuric acid after 10 mins. Plate readout was at 450 nm. The reference wavelength of 570 nm was used (values read at 570nm were subtracted from those read at 450nm, giving the absorbance value).

H. bakeri infection intensity of offspring. On PD 27, ten offspring per sex from five *H. bakeri* infected and five uninfected dams were infected using an oral gavage needle with 150 ± 3 L3 *H. bakeri* suspended in 0.1 mL distilled water. At 36 days post infection, for a 20 hr period, each mouse was placed into an individual wire-bottomed cage which allowed for collection of fecal pellets. Drinking water was provided *ad libitum* but food was withheld. Fecal pellets were collected and the McMaster technique was used to determine egg production expressed as eggs per gram of faeces (EPG). At 38 days post infection, mice were euthanized using isoflurane, followed by CO2, and intestines were collected. The number of male and female worms were counted (i.e. worm burden) and the fecundity of female worms of *H. bakeri* were determined as parasitological indicators of infection intensity.

Statistics. Statistical analyses were performed in R statistical software 4.2.3, and figures were produced using GraphPad Prism V9. To assess *H. bakeri*-specific IgG1 antibody absorbance, where we had repeated measures across different dilution factors, models were built with maternal treatment condition (*H. bakeri* infected vs. uninfected), offspring sex (male vs. female) and dilution factor as fixed factors, and the identity of the mouse as a random factor. EPG, fecundity and worm burden, models were built with maternal treatment condition (*H. bakeri* infected versus uninfected) and offspring sex (male versus female) as fixed factors, and dam as a random factor to account for pseudoreplication. Non-significant interactions between fixed effects were excluded from models.

Linear mixed models were built to assess *H. bakeri*-specific IgG1 antibody absorbance, EPG and fecundity using the lme function in the nlme package[94]. As worm burden was a

discreate and overdispersed variable, a negative binomial generalized linear model was built using the glmer.nb function in the lme4 package[95]. Where necessary, post hoc pairwise comparisons were performed using the emmeans function (emmeans package[96]) with a Tukey correction. Normality, independence and homogeneity of variances of mixed models were assessed using fitted residuals from the plotresid function (RVAideMemoire package[97]), and the DHARMa package[98]. Values are presented as means ± SEM. The significance level was set at 0.05.

Neuroimmune study.

Tissue preparation. On PD 22, six offspring per sex from *H. bakeri* infected or uninfected dams (no more than one pup/sex/dam) were anesthetized with intraperitoneal injection of avertin (600 mg kg^{-1} body weight) and then transcardially perfused with ice cold 1 x PBS followed by 4% paraformaldehyde in PBS w/v. Collected brains were fixed with 4% PFA overnight and cryoprotected in 30% sucrose for 24 hr. Brains were then embedded in OCT medium and stored at -80°C. Using a cryostat (Leica CM3050 S), serial coronal sections (20 µm thick) of the brain were obtained, with a focus on the dorsal hippocampus, and mounted on microscope slides.

Immunohistochemistry. Hydrophobic pen (ImmEdge Pen, Vector Laboratories) was used to create a water-repellent barrier to keep reagents localized on tissue sections. Tissue sections were hydrated in 1× PBS and then incubated in blocking solution (0.03% Triton X-100, 3% heat-induced horse serum (HIHS) and 3% BSA in 1× PBS) for 1 hr at RT. Primary antibody was diluted in new blocking solution, added to the tissue sections and incubated at 4 °C overnight. Tissue sections were washed with 1× PBS three times for 10 min each. Secondary antibody was diluted in 3% HIHS, 3% BSA in 1× PBS and added to the tissue sections and incubated for 1 hr at RT. These steps were then repeated for the second and third primary and secondary antibodies. Once the final secondary antibody had been applied, tissue sections were washed with 1× PBS for 10 min followed by staining with Hoechst (1:10,000) in 1× PBS for 10 min. Two additional 10 min washes in 1× PBS were performed before tissue sections were air dried and mounted (Dako Fluorescence Mounting Medium, S3023; Agilent). Negative controls that omitted the primary antibodies were included.

Antibodies. The following antibodies were used for immunofluorescent staining: Glial Fibrillary Acidic Protein (GFAP) antibody (AB5541, 1:500; Millipore) to detect astrocytes; Ionized Calcium Binding Adapter Molecule 1 (Iba1) antibody (019-19741, 1:500; Wako) to detect microglia. We also stained with Mouse Macrophage Mannose Receptor/CD206 antibody (AF2535, 1:40; R&D Systems), a cell surface protein that is typically increased in response to Th2 cytokines[30,31,35]. This allowed us to detect double stained Iba1+/CD206+ cells. For secondary antibodies, we used Alexa Fluor 647 Goat Anti-Chicken IgY (A21449, 1:500; ThermoFisher Scientific), Alexa Fluor 555 Donkey Anti-Rabbit IgG (A31572, 1:500; ThermoFisher Scientific) and Alexa Fluor 488 Donkey Anti-Goat IgG (A11055, 1:500; ThermoFisher Scientific).

Image capture and analysis. Confocal microscope (Leica SP8) was used to image three dorsal hippocampus sections/animal with a focus on the CA3-CA1 region (Supplementary Fig. 3), as this hippocampal region has been shown to play an important role in the encoding and retrieval of spatial memories[99,100]. In ImageJ, three 0.1 mm^2 boxes were drawn with the same reference position of the hippocampus proper for each section (Supplementary Fig. 3). The numbers of astrocytes (GFAP+), microglia (Iba1+), and CD206 positive microglia (Iba1+/CD206+) in each box were counted.

Statistics. The total number of cells in all nine boxes was calculated per mouse to provide cell density (# cells/ 0.9 mm^2). The percentage of CD206 positive microglia relative to the total number of microglia cells was calculated. Two-way ANOVAs were performed in GraphPad Prism (Version 10.0.2), to determine the effect of maternal *H. bakeri* infection and offspring sex on microglial and astrocyte cell density. Values are presented as means ± SEM. The significance level was set at 0.05.

Data Availability

The datasets generated and analysed for the long-term potentiation, resistance and neuroimmune study are available via a link to the Borealis Dataverse [https://doi.org/10.5683/SP3/3NQFPS], a public data repository. The dataset generated and analysed for the gene expression study was deposited in the National Center for

Biotechnology Information Sequence Read Archive Database [BioProject: PRJNA1071490; https://www.ncbi.nlm.nih.gov/sra/PRJNA1071490].

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Author Contributions

S.C.N. conceived and designed the study, conducted experimental work, analyzed the data, interpreted the results, and drafted the manuscript. For the gene expression study, J.E. performed the raw data processing of the FASTQ files, assisted with the bioinformatics analysis and provided helpful suggestions for the manuscript. For the LTP study, A.J.M. trained and supervised J.F.M. and S.C.N., and J.F.M. performed the electrophysiology and assisted with data extraction. J.M.G. trained S.C.N. for the IHC portion of the neuroimmune study (tissue preparation, immunohistochemistry and image capture and analysis). M.E.S., T.E.K. and A.J.M. provided input on study design, data interpretation and feedback and suggestions for the manuscript. M.E.S. and T.E.K. obtained funding for the research.

Competing Interests

The authors declare no competing interests.

Supplementary Information

Supplementary figures are included below, however, six supplementary tables also accompany this manuscript. These tables are located in a single excel file which can be downloaded from the "Supplementary Information" section at: https://doi.org/10.1038/s41598-024-60865-2

Figures

Figure 1. Maternal *H. bakeri* infection influenced offspring hippocampal gene expression**. (a)** Principal component analysis (PCA) showing the relationship between the filtered and normalized gene expression profiles of hippocampal samples from offspring born to *H. bakeri* infected dams (blue) and uninfected dams (black). **(b)** Volcano plot indicating 2166 up-regulated (red, adjusted p-value < 0.05, FDR method) and 2171 down-regulated (blue, adjusted p-value < 0.05, FDR method)) genes in the hippocampus of offspring in response to maternal *H. bakeri* infection. Genes with an adjusted p-value > 0.05 are in grey. The x-axis is log2FC and the y-axis is -log10(p-value). n = 9-10 offspring per maternal treatment condition.

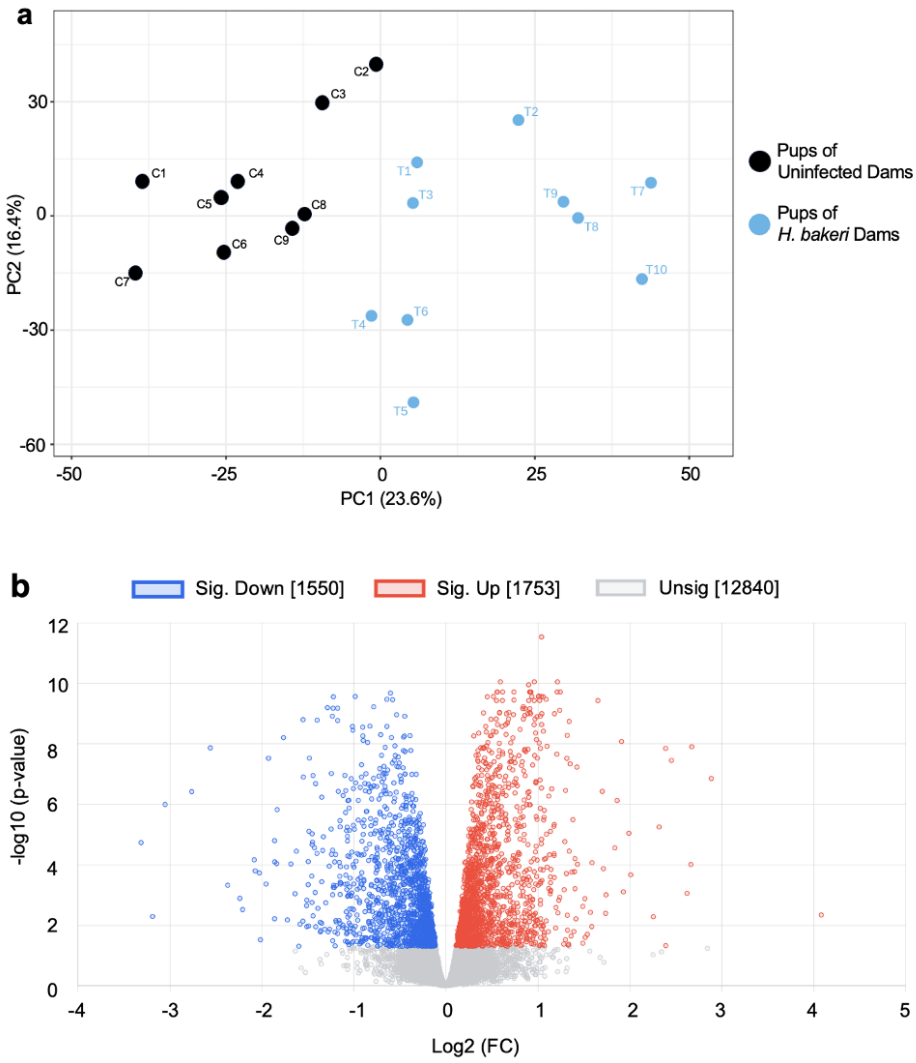


Figure 2. Maternal *H. bakeri* infection increased the expression levels of long-term potentiation and TGF-β signaling KEGG pathways in offspring hippocampus. Heat map of up- and down-regulated genes involved in **(a)** long-term potentiation KEGG pathway (FDR = 0.02) and **(b)** TGF-β signaling KEGG pathway (FDR = 0.03). Blue and red cells correspond to lower and higher expression levels, respectively. n = 9-10 offspring per maternal treatment condition.

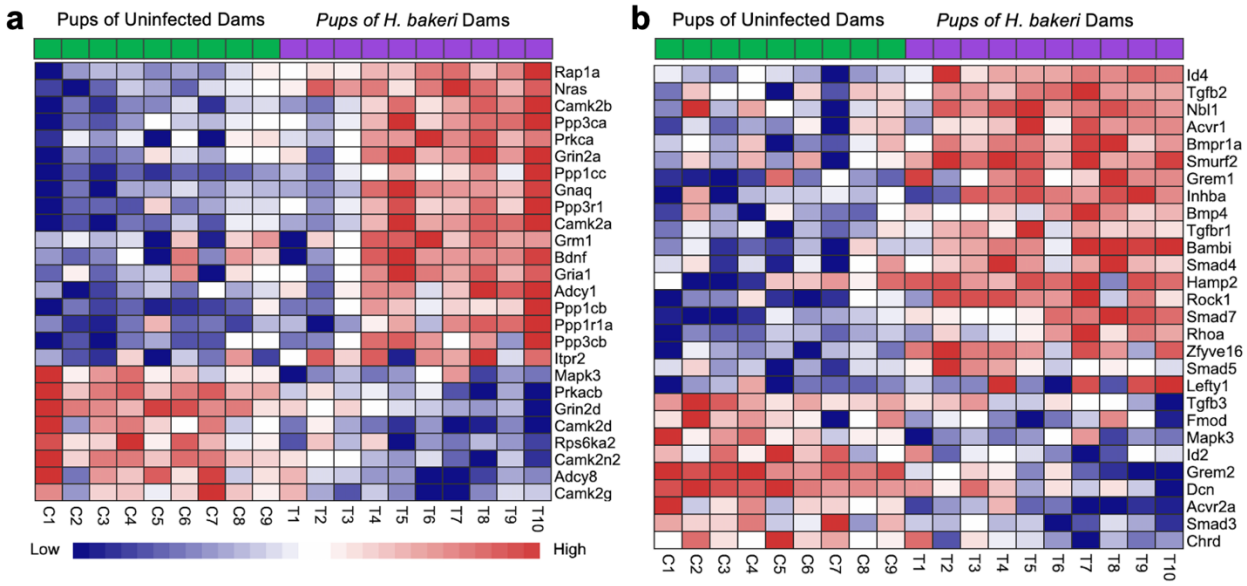


Figure 3. Maternal *H. bakeri* infection increased the expression of genes associated with microglia (M), astrocyte (A) and oligodendrocyte (O) markers in offspring hippocampus. n = 9-10 offspring per maternal treatment condition.

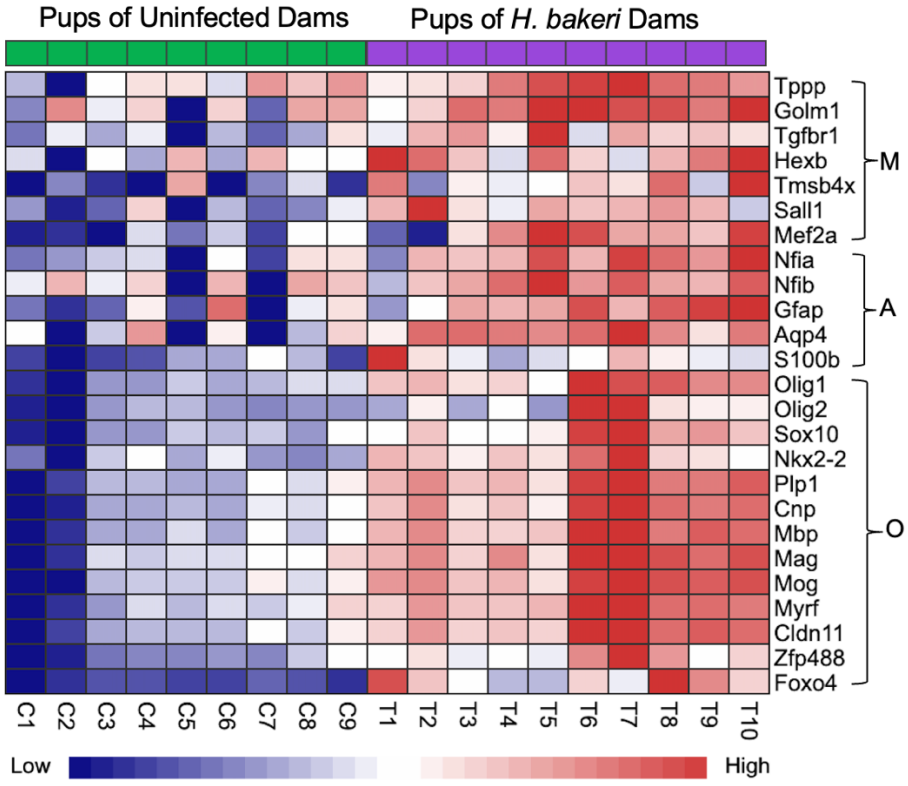


Figure 4. Maternal *H. bakeri* infection enhanced long-term activity-dependent synaptic plasticity at hippocampal CA3-CA1 Schaffer collateral synapses of three week-old male offspring. **(a)** Input/output curves showed no differences in basal synaptic transmission between groups. **(b)** Paired-pulse facilitation ratio (PPF) was not altered by maternal *H. bakeri* infection. PPF was obtained by delivering two stimuli with increasing interpulse intervals. PPF was calculated by dividing the second peak slope by the first. **(c)** High-frequency stimulation (HFS)-induced LTP at CA1 was enhanced in juvenile offspring born to *H. bakeri* dams relative to offspring born to uninfected dams (red arrows indicate HFS). **(d)** fEPSP slope measured 60 min after the HFS, indicating maintenance of LTP in 5 of 7 offspring born to *H. bakeri* dams and to only 1 of 7 offspring born to uninfected dams. Values are means±SEM, n = 7 offspring per maternal treatment condition (*$p < 0.05$).

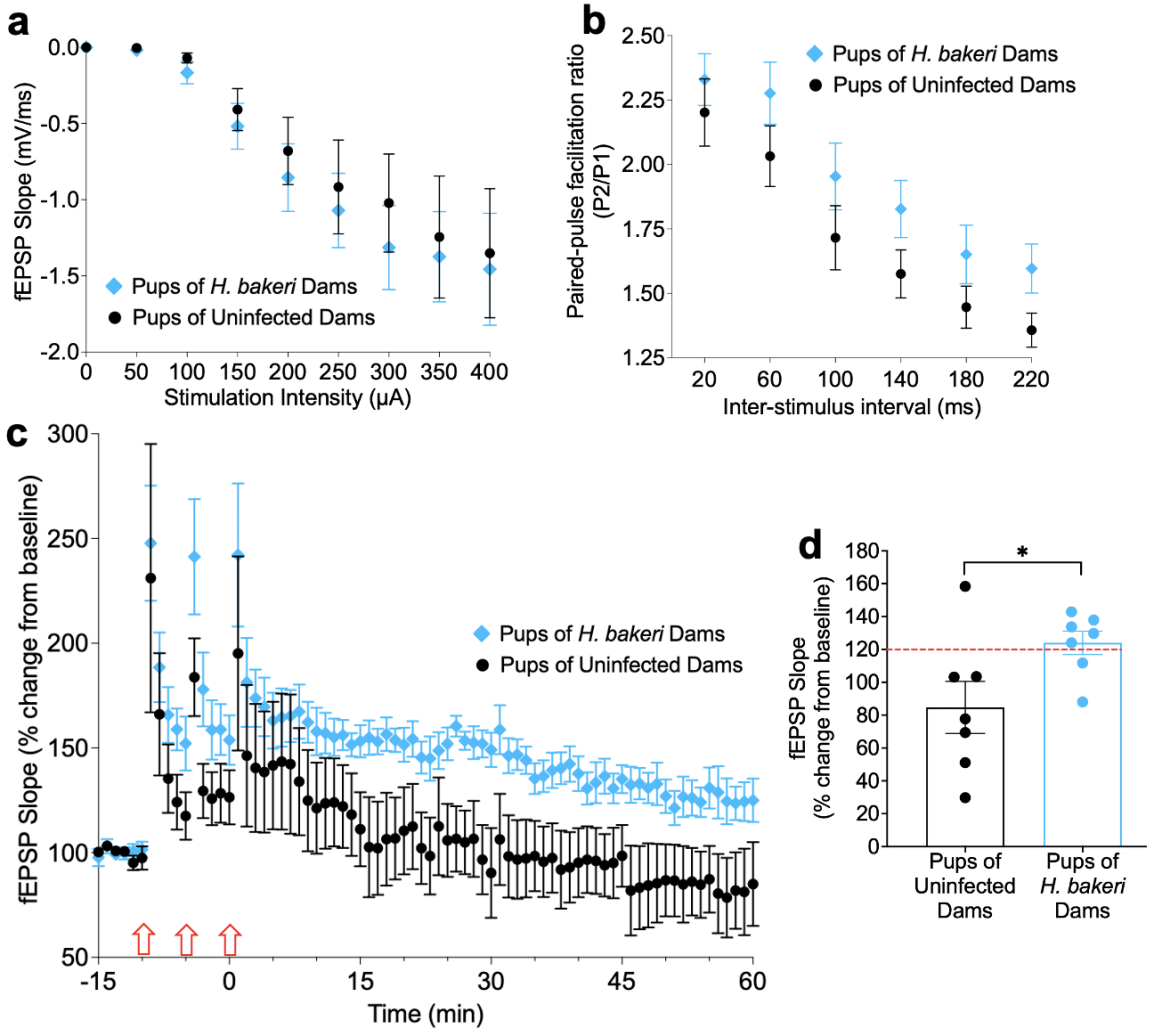


Figure 5. Maternal *H. bakeri* infection resulted in greater resistance to *H. bakeri* infection of their offspring consistent with maternal transfer of parasite-specific IgG1. **(a)** At PD 24, serum was collected from uninfected offspring of *H. bakeri* infected and uninfected dams and *H. bakeri*-specific IgG1 antibody absorbance curves were obtained via ELISA. Uninfected pups of *H. bakeri* infected dams (n = 8) had detectable levels of *H. bakeri*-specific IgG1 antibody while pups of uninfected dams (n = 4) did not. (b-d) At PD 27, ten male and ten female offspring of *H. bakeri* infected and uninfected dams were infected with 150 *H. bakeri* larvae, and one-month later, **(b)** eggs per gram of faeces, **(c)** worm burden and **(d)** parasite fecundity were obtained as parasitological indicators of infection intensity. *H. bakeri* infection intensity was lower in pups of *H. bakeri* dams compared to pups of uninfected dams. Since no significant sex differences were found between pups, pooled data is shown. Values are means±SEM, n = 20 offspring per maternal treatment condition (**$p < 0.01$, *** $p < 0.001$).

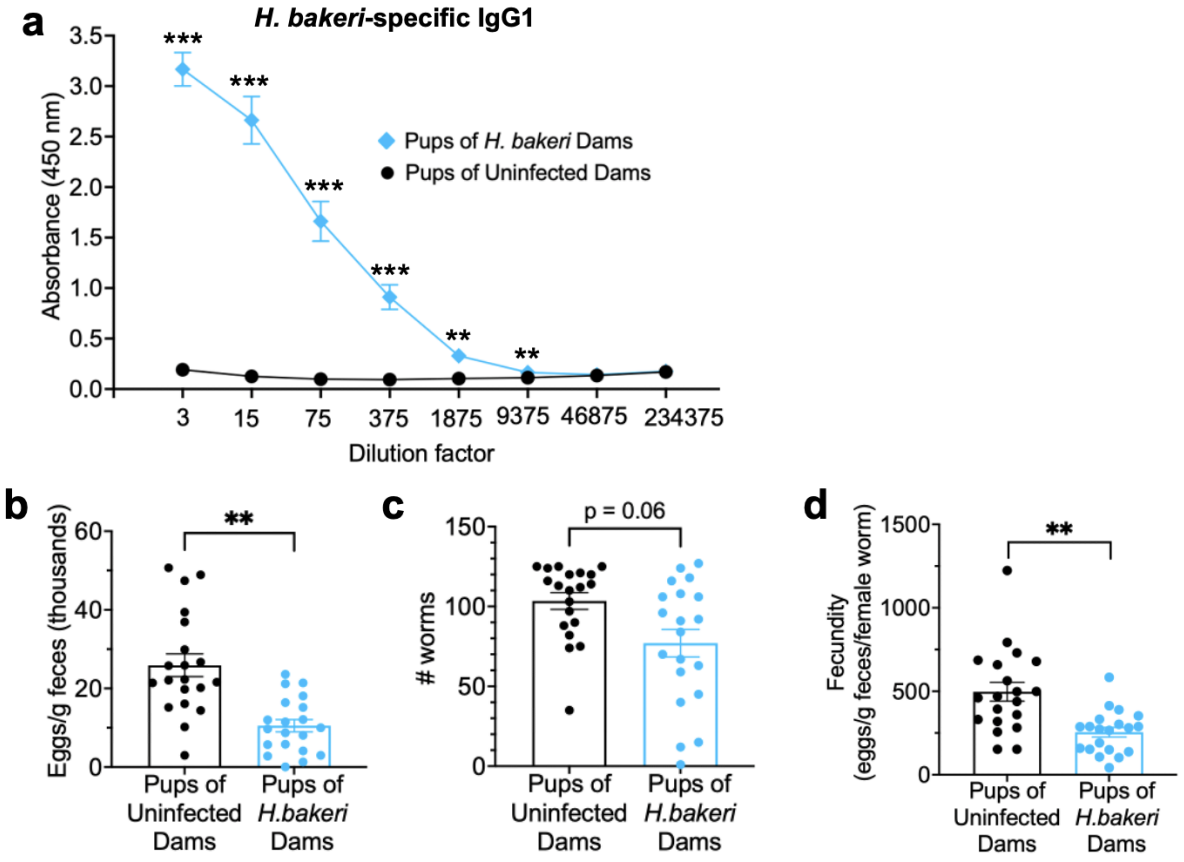


Figure 6. Astrocyte and microglia density are increased in hippocampus of offspring born to *H. bakeri* infected dams. **(a)** Astrocytes were detected with an antibody directed against GFAP (magenta) and **(b)** microglia were detected with an antibody directed against Iba1 (red). Cells within 0.1 mm^2 yellow boxes (shown below) were counted. Cell nuclei were stained with Hoechst dye (cyan). Scale bar: 50 µm. **(c)** GFAP positive cells and **(d)** Iba1 positive cells were counted in three x 0.1 mm^2 boxes per hippocampal section, and a total of three hippocampal sections per mouse were assessed (# cells/ 0.9 mm^2/mouse). Since no significant sex differences were evident, pooled female and male data are shown. Values are mean ± SEM, n = 12 offspring per maternal treatment condition (*$p < 0.05$, **$p < 0.01$).

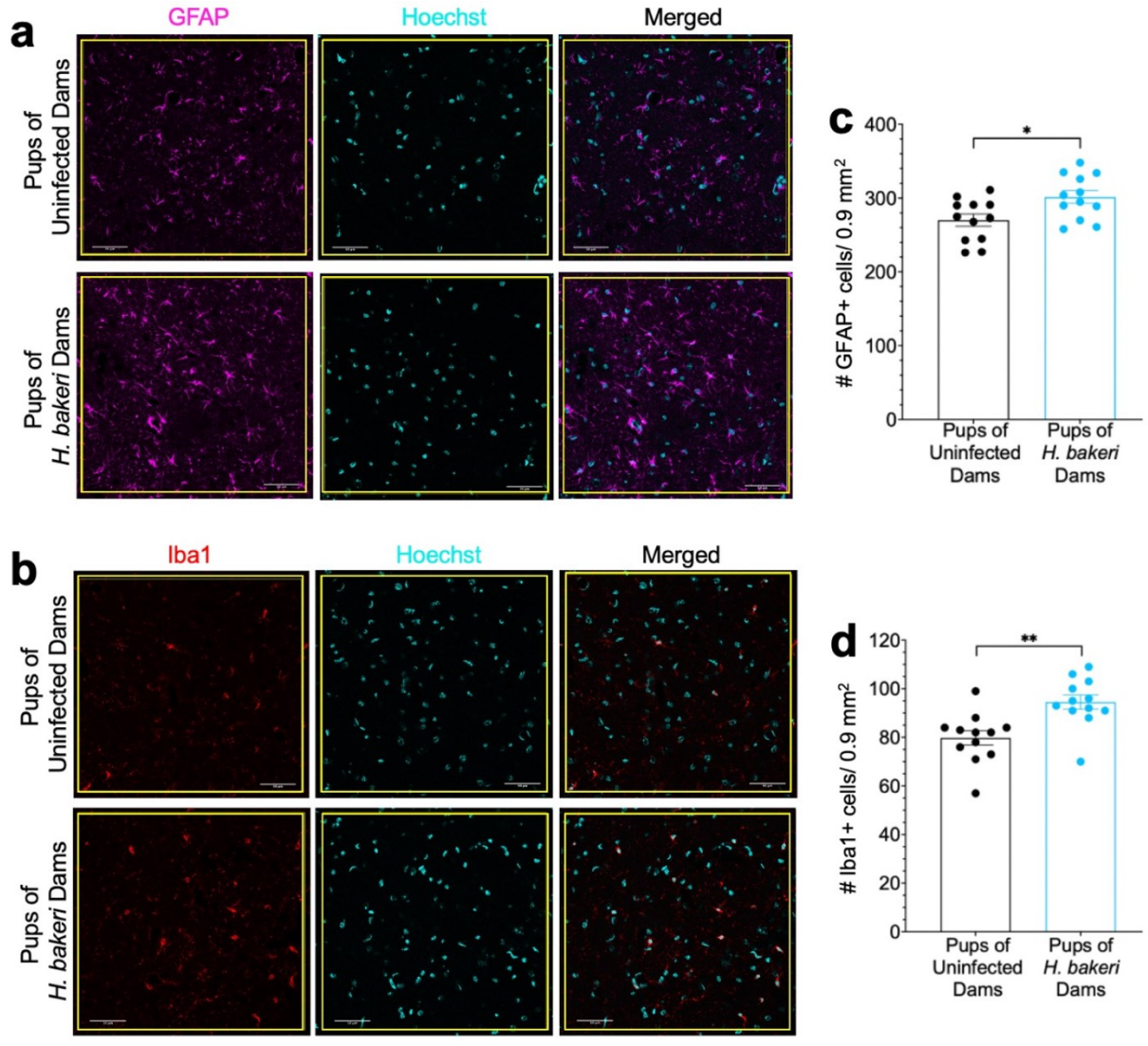
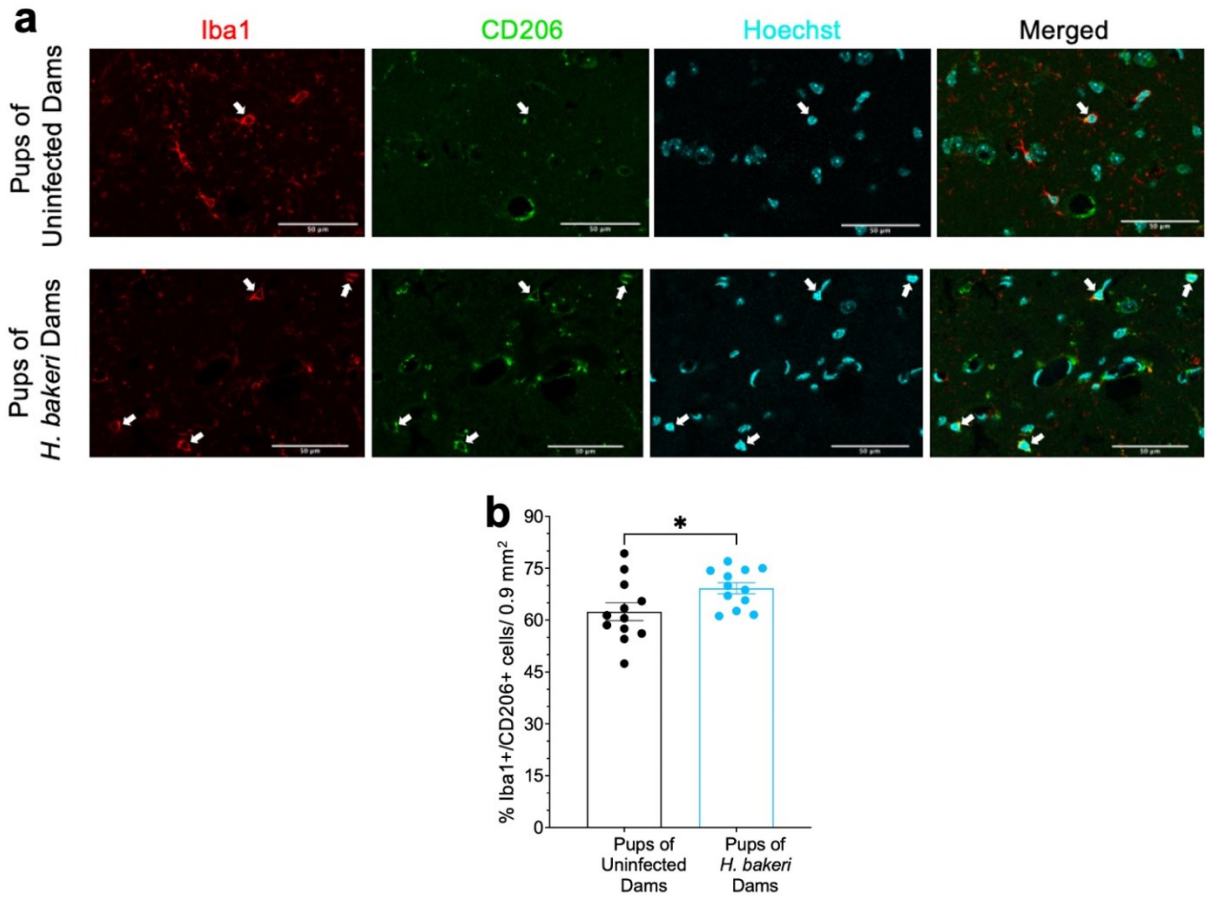
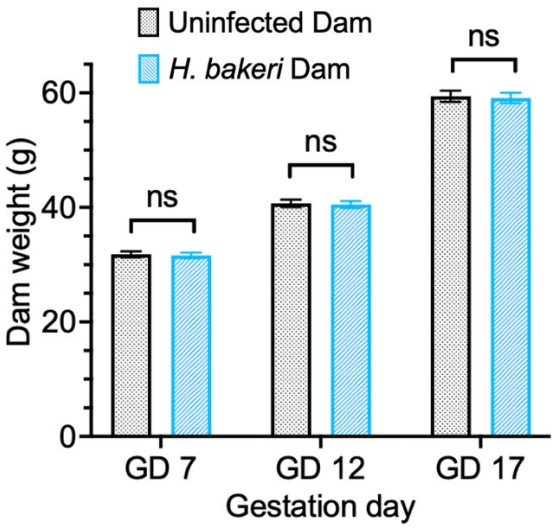


Figure 7. Percent of microglia positive for CD206 is increased in hippocampus of offspring born to *H. bakeri* infected dams. (**a**) Double immunofluorescence labeling of Iba1 + /CD206 + microglia cells in hippocampus of offspring born to *H. bakeri* infected or uninfected dams. Microglia were detected by Iba1 antibody (red). To detect CD206 positive microglia, the Mouse Macrophage Mannose Receptor/CD206 antibody was used (green), and double labelled Iba1 + /CD206 + cells were assessed (indicated by white arrows). Cell nuclei were stained with Hoechst dye (cyan). Scale bar: 50 µm. (**b**) All Iba1 + and Iba1 + /CD206 + cells were counted in three × 0.1 mm^2 boxes per hippocampal section, and a total of three hippocampal sections per mouse were assessed (# cells/0.9 mm^2/mouse). The percent of Iba1 + /CD206 + cells relative to the total number of Iba1 + cells was calculated. Since no significant sex differences were evident, pooled female and male data are shown. Values are mean ± SEM, $n = 12$ offspring per maternal treatment condition (*$p < 0.05$).

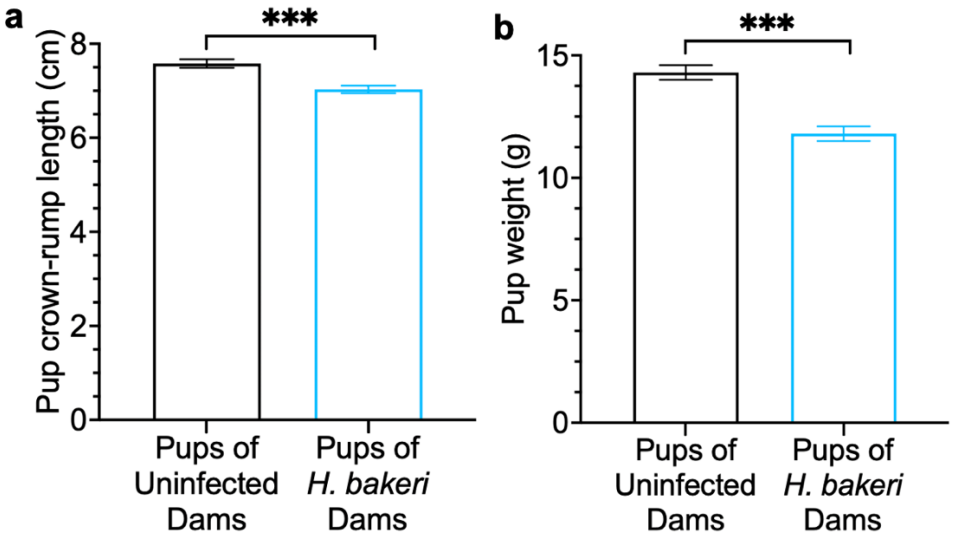


Supplementary Figures

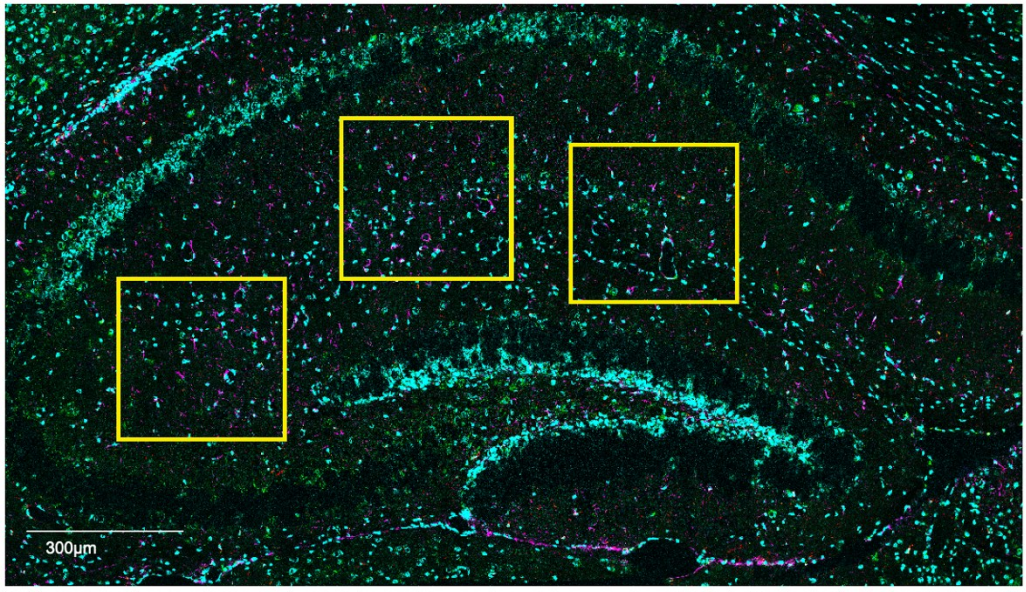
Supplementary Figure 1. Maternal *H. bakeri* infection did not influence dam weight at gestation day (GD) 7, 12 and 17. The identity of the dam was included as a random factor and litter size as a covariate. Values are LSmeans±SEM, n = 18-20 per group (ns = not significant).



Supplementary Figure 2. Pups born to *H. bakeri* infected dams had shorter length and lower mass than pups of uninfected dams at postnatal day 20. Pups were nested within dam, and offspring sex and litter size were included as covariates. Values are LSmeans±SEM, n = 54-59 offspring per group (***$P < 0.001$). **(a)** pup crown-rump length and **(b)** pup body mass.



Supplementary Figure 3. Immunofluorescence labeling of dorsal hippocampus to assess density of astrocytes, microglia, and CD206 positive microglia in response to maternal *H. bakeri* infection. Astrocytes were detected by glial fibrillary acidic protein (GFAP) antibody (magenta) and microglia were detected by ionized calcium binding adapter molecule 1 (Iba1) antibody (red). To detect CD206 positive microglia, the Mouse Macrophage Mannose Receptor/CD206 antibody was used (green), and double labelled Iba1+/CD206+ cells were assessed. Cell nuclei were stained with Hoechst dye (cyan). Confocal microscope (Leica SP8) was used to image three dorsal hippocampus sections/ animal. In ImageJ, three 0.1 mm^2 boxes were drawn with the same reference position of the hippocampus proper for each section (shown in yellow). The numbers of astrocytes (GFAP+), microglia (Iba1+), and CD206 positive microglia (Iba1+/CD206+) in each box were counted and summed to provide cell density (# cells/ 0.9 mm^2/mouse). Scale bar: 300 µm.



Connecting statement II

In Chapter IV, I examined the effect of a GI nematode infection during pregnancy and lactation on offspring hippocampal LTP, gene expression and neuroimmunity, and resistance to direct infection as as an index of peripheral immunity. The gene expression and electrophysiological data indicated enhanced hippocampal LTP in three-week old uninfected offspring. Furthermore, maternal infection improved resistance to direct infection of *H. bakeri* in juvenile offspring, correlated with transfer of parasite-specific IgG1 to their serum. Finally, at three-weeks of age, hippocampal immunohistochemistry and gene expression data suggested a Th2/Treg biased neuroimmune environment in male and female uninfected offspring of infected mothers, mimicking the peripheral immune response observed in the *H. bakeri* infected mother [8,9].

This is the first study to show that a maternal GI nematode infection promotes hippocampal LTP in the uninfected juvenile offspring, possibly through transfer of a Th2/Treg immune phenotype from the infected dam that protects the offspring from direct infection and extends to their developing brain. These findings highlight a potential mechanism underlying my previous observations in Chapter III of enhanced spatial memory in two and three week-old uninfected male and female offspring in response to maternal *H. bakeri* infection.

Previous studies have identified that maternal immune activation with bacterial or viral pathogens or their mimics, can lead to chronic neuroinflammation in offspring, underlying long-lasting impairments in hippocampal function and cognitive deficits that progress into adulthood [58,275]. As such, I was interested in assessing cognitive function of offspring at three-months of age, as this is when brain maturation is complete[163]. Therefore, in Chapter V, I determined if the positive impact of maternal *H. bakeri* infection during pregnancy and lactation on spatial memory of juvenile offspring was retained in adult offspring.

NOTE: References for Connecting statement II can be found in the "Master List of References for All Non-Manuscript Sections" located at the end of the document.

Chapter V - Gastrointestinal nematode infection during pregnancy and lactation enhances spatial reference memory and reduces indicators of anxiety-like behaviour in uninfected adult female mouse offspring

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Abstract

Maternal bacterial and viral infections that induce neuroinflammation in the developing brain are associated with impaired cognitive function and increased anxiety in the offspring. In contrast, maternal infection with the immunoregulatory murine gastrointestinal (GI) nematode, *Heligmosomoides bakeri*, appears to benefit neurodevelopment as juvenile two and three week-old male and female offspring had enhanced spatial memory, which may be due to a Th2/Treg biased neuroimmune environment. Here, the impact of maternal *H. bakeri* infection during pregnancy and lactation on the spatial and anxiety-like behaviours of adult, three month-old uninfected male and female offspring was explored for the first time. It was observed that adult female offspring of *H. bakeri* infected dams had enhanced spatial reference memory and reduced anxiety-like behaviour compared to females of uninfected dams. These effects were not observed in adult male offspring. Thus, the positive influence of a maternal GI nematode infection on spatial memory of juvenile offspring persists in adult female offspring.

Introduction

Brain development is a highly plastic process that, in rodents, starts *in utero* and continues postnatally until three months of age when brain maturation is completed (Hammelrath et al., 2016). During this vulnerable period, environmental stimuli such as maternal physical exercise favor brain development (Robinson and Bucci, 2014; Gomes da Silva et al., 2016), whereas exposure to bacterial and viral infections or toxins during pregnancy impairs neurodevelopment (Boksa, 2010; Wilhelm and Guizzetti, 2015; Bergdolt and Dunaevsky, 2019; Beversdorf et al., 2019). There has been a large effort over the past two decades to understand the link between maternal exposure to these pathogens and the risk of neurological disorders in offspring with a developmental origin, including autism spectrum disorder and schizophrenia (Boksa, 2010; Bergdolt and Dunaevsky, 2019). With the help of rodent models, it is now understood that the maternal immune response, not a specific pathogen, is a risk factor for neurodevelopmental disorders (Bergdolt and Dunaevsky, 2019). Viral or bacterial pathogens or their mimics (polyinosinic-polycytidylic acid [Poly I:C] or lipopolysaccharide [LPS] respectively) induce a strong pro-inflammatory immune response in the mother, which extends to the offspring, resulting in an altered immune profile in the developing brain which ultimately leads to irreversible neurodevelopmental defects and the emergence of behavioral abnormalities and cognitive impairments (Boksa, 2010; Bergdolt and Dunaevsky, 2019). Interestingly, however, it was observed that maternal exposure to the immunoregulatory gastrointestinal (GI) nematode, *Heligmosomoides bakeri* (also referred to as *Heligmosomoides polygyrus* and previously known as *Nematospiroides dubius*), may actually benefit, not harm, at least some aspects of brain development of the offspring (Haque et al., 2019; El Ahdab et al., 2021; Noel et al., 2022; Noel et al., 2024). In contrast to the type 1 pro-inflammatory immune responses (e.g. IFN-γ, TNF-α, CD4+ T helper type 1 (Th1) cells) triggered by bacterial or viral infections, similar to most GI nematodes, infection with *H. bakeri*, elicits a type 2 host resistance and tolerizing immune response (e.g., IL-4, IL-5, IL-13, Th2 cells) (Maizels et al., 2012; Reynolds et al., 2012; Chen et al., 2023). *H. bakeri* also induces an immunoregulatory network that aids long-term survival in its host, involving proliferation of Foxp3+ CD4+ regulatory T (Tregs) cells and the potent immunoregulatory cytokines IL-10 and TGF-β (Maizels et al., 2012; Reynolds et al., 2012). This also allows the nematode to dampen inflammatory and pathologic processes, and

prevent or ameliorate a number of hyper-immune/inflammatory diseases (Elliott et al., 2004; Wilson and Maizels, 2006; Saunders et al., 2007; Smallwood et al., 2017; White et al., 2020).

Brain gene expression in seven-day old neonatal male offspring born to *H. bakeri* infected CD-1 outbred dams revealed up-regulation of five key interacting pathways associated with long-term potentiation (LTP) (Haque et al., 2019), the cellular mechanism of learning and memory (Dong et al., 2015). This was consistent with gene expression and electrophysiological data indicating enhanced hippocampal LTP in three-week old uninfected offspring (Noel et al., 2024). The hippocampus is involved in cognitive functions, and also plays an important role in the regulation of emotional behaviors, particularly anxiety (Ghasemi et al., 2022). Spatial learning and memory in rodents are critically dependent on hippocampal synaptic plasticity (Clark and Martin, 2005; Dringenberg, 2020), thus, enhanced hippocampal LTP was consistent with the enhanced spatial memory that was observed in two and three-week old uninfected male and female offspring of *H. bakeri* infected dams (Noel et al., 2022). Furthermore, maternal *H. bakeri* infection resulted in a Th2/Treg biased neuroimmune environment in the uninfected offspring. Gene expression analysis of the brains of neonates of infected dams revealed up-regulated Th2/Treg pathways, including the genes for the potent immunoregulatory cytokines IL-4 and TGF-β, and down-regulated Th1/Th17 pathways (Haque et al., 2019; El Ahdab et al., 2021), mimicking the immune response of the infected mother (Odiere et al., 2013; Su et al., 2023). This altered neuroimmune environment was also seen in the hippocampus of male and female three-week old juvenile offspring where the immunoregulatory TGF-β signaling pathway was up-regulated, and where a greater number of two immune sensitive cells, microglia and astrocytes, were observed, as well as a higher percentage of CD206 positive microglia (Noel et al., 2024) which are typically increased in response to the Th2 cytokine, IL-4 (Francos-Quijorna et al., 2016; Liu et al., 2016; Jurga et al., 2020; Zhang et al., 2021). These findings reveal a potential mechanism behind the enhanced LTP and spatial memory of uninfected offspring, as both TGF- β and IL-4 are known to have measurable downstream effects on LTP and spatial memory (Nolan et al., 2005; Fukushima et al., 2007; Derecki et al., 2010; Gadani et al., 2012; Caraci et al., 2015). Whether the improved spatial memory extends to adult offspring is, however, unknown.

The Barnes Maze Test (BMT) has been previously used to assess spatial learning and memory of three-week old offspring in response to maternal *H. bakeri* infection (Noel et al., 2022). This test assesses hippocampus-dependent spatial reference memories formed over repeated trials in an unchanging environment (Sharma et al., 2010) by measuring the ability of rodents to learn and recall the location of a goal box which is located under one of 20 holes around the perimeter of a platform (Sharma et al., 2010). It involves a habituation trial (Day 0), a training phase (Days 1–4) to test spatial learning, both of which contain the goal box, and probe trials 1 (Day 5) and 2 (Day 12), with no goal box, to test short-term and long-term spatial reference memory respectively. Of note, although the BMT is primarily designed to assess spatial learning and memory, it can also be used to explore two indicators of anxiety-like behavior. First, when rodents are initially placed in an environment that is distinctly different from any environment they have previously encountered, they become afraid or anxious, and defecate (Denenberg, 1969; Bailey and Crawley, 2011). Thus, quantifying the number of fecal pellets during the five min habituation trial when mice are first introduced to the BMT can be used as an indicator of their anxiety/fear level, with a greater number of fecal pellets indicating a higher level of anxiety/fear (Denenberg, 1969; Bailey and Crawley, 2011). Second, mice tend to favor darker, more enclosed spaces and thus avoid exploring open areas, especially when they are brightly lit (Bailey and Crawley, 2011). Thus, during the BMT training trials, mice are motivated to seek shelter from the brightly lit, exposed maze, by entering the dark enclosed goal box. It can therefore be interpreted that mice that find the goal box, but choose instead to continue exploring the maze, may be less fearful/anxious than mice who immediately enter the goal box upon finding it.

Using the BMT, the goal of this study was to determine if the positive impact of maternal *H. bakeri* infection during pregnancy and lactation on spatial memory of juvenile offspring was retained in adult offspring. Indicators of anxiety-like behavior in these offspring were also explored. This study presents the first evidence that enhanced spatial memory previously observed in juvenile offspring in response to a maternal GI nematode infection is retained in uninfected adult female, but not male, offspring, and that these adult females also have reduced anxiety-like behaviour compared to female offspring of uninfected dams.

Materials and Methods

Experimental design

A 2×2 factorial design was employed using *H. bakeri* infected versus uninfected dams, and their male versus female offspring. All procedures were approved by the McGill University Animal Care Committee according to the guidelines of the Canadian Council on Animal Care.

Mice and Parasites

Of the 40 primiparous 8-week-old timed pregnant outbred CD-1 mice that were received from Charles River Laboratories (Quebec, Canada) on gestation day [GD] 4 , 31 were pregnant (78% pregnancy rate). Each dam with her litter was housed individually in a Nalgene cage (Fisher Scientific, Canada) at 21–23 °C, 40–60% relative humidity and a 12 h light and dark cycle. Mice had *ad libitum* access to a 2920X Teklad rodent diet (18% crude protein, 5% crude fat, 5% crude fiber). Within each of the six staggered groups of dams received over 3 months, dams were randomized into uninfected and infected groups, providing a total of 15-16 dams per group were used for this study. This provided an acceptable sample size based on a minimum of at least six dams per treatment condition (Meyer et al., 2009). Using standard *H. bakeri* protocols (Johnston et al., 2015), infective L3 were obtained by fecal culture of stock parasites maintained in outbred CD-1 mice. In our previous studies on the impact of maternal *H. bakeri* infection on uninfected offspring (Haque et al., 2019; Noel et al., 2022; Noel et al., 2024), a trickle infection protocol was used to maximize antigenic stimulation during pregnancy and lactation and to simulate ongoing natural infection that occurs in wild mice (Brailsford and Behnke, 1992). This same protocol was used here. Dams in the infected group were intubated using an oral gavage needle with 100 ± 3 L3 suspended in 0.1 mL distilled water on GD 7, 12, 17, and postnatal day (PD) 3, 8 and 13 (Fig. 1). Uninfected dams were intubated at the same frequency with 0.1 mL distilled water, to control for any stress due to handling. Given that *H. bakeri* eggs released into the environment develop into infective larvae after 7 days, all cages were cleaned every 5 days to ensure offspring could not ingest infective larvae. Dams were weighed on GD 7, 12 and 17. Following weaning (PD 20), dams were euthanized and necropsied to confirm successful infection of dams based on the presence of adult worms in the small intestine.

Pups were born on GD 19, litter size was recorded on PD 3, 8, 13 and 20, and body mass and length from the top of the head to the base of the tail were recorded on PD 20 and 69. At PD 20, pups were weaned, sexed and given a unique identifier with a permanent marker, and one male and one female pup per litter were randomly selected for the Barnes Maze Test (BMT) (Fig. 1). Pups selected for the adult BMT were housed with two littermates of the same sex until testing was performed 2 months later. After the BMT, experimental pups were necropsied and intestines were examined for adult *H. bakeri* to confirm that they had not accidentally become infected. Remaining pups were used for a separate experiment (Noel et al., 2024).

Experimental Room

The BMT was conducted in a quiet room (340 cm × 260 cm) which was brightly lit (a floor lamp in each corner and an over-head light) to provide a mild negative reinforcement. Trials were recorded using an overhead monochromatic video camera (Basler Ace monochrome) connected to a computer located in the back corner of the room behind a curtain. The experimenter remained behind the curtain during all recordings. Data was extracted from the videos using the Ethovision XT software (version 17). All equipment remained in the same location in the room, providing visual spatial cues.

Barnes Maze Test

The BMT procedure followed a protocol (Sunyer et al., 2007) that was previously used to successfully test spatial learning and short and long-term reference memory in juvenile CD-1 mice born to uninfected or *H. bakeri* infected dams (Noel et al., 2022), as well as adult CD-1 mice born to uninfected dams (Patil et al., 2009). As previously described (Noel et al., 2022), the Barnes Maze (Maze engineers, 412 Wilmette Ave, Glenview, IL 60025, USA) is an opaque circular platform (diameter: 92 cm, height: 70 cm) with 20 equally spaced holes (diameter: 5 cm) located 2 cm from the edge. In a brightly lit environment, mice naturally seek the dark enclosed area provided by the black goal box (20 × 10 × 4 cm) located under the same escape hole throughout all trials. From the surface of the maze, the escape hole, containing the goal box,

looks identical to the other 19 holes. Mice learn the location of the goal box based on spatial cues in the room.

The BMT was conducted when offspring were 3 months old. The BMT involved a habituation trial (Day 0), a training phase (Days 1–4) to test spatial learning, and probe trials 1 (Day 5) and 2 (Day 12) to test short-term and long-term spatial reference memory, respectively. Home cages were moved into the experimental room for 15–20 min acclimation prior to trials and all equipment was cleaned with 70% ethanol between trials.

The habituation trial was used to introduce the mouse to the apparatus and reduce anxiety during the test. The mouse was placed in an opaque starting cylinder (diameter: 10.5 cm, height: 8 cm) at the center of the platform. After 10 s, the cylinder was removed, and the animal was allowed to freely explore the apparatus for 5 mins. After 5 min, the mouse was guided to the goal box and remained there for 2 min. During the habituation trial, the number of fecal droppings was counted, as an indicator of anxiety (Denenberg, 1969).

Training involved four 3 min trials per day for four training days. Each of the 16 training trials began by placing the mouse in the starting cylinder at the center of the platform. After 10 s, the cylinder was removed, recording began, and the animal was allowed to freely explore the apparatus for 3 min. Once the animal entered the goal box, it was allowed to remain there for 1 min. Mice that failed to enter the goal box within 3 min were gently guided to its location and placed inside. After each of the four 3 min training trials per day, mice were returned to their home cage for 20 min. During the training trials, the following variables were recorded: (1) Total latency (s), defined as time taken until the mouse enters the goal box; (2) Total distance (cm), defined as distance travelled until the mouse enters the goal box; (3) Total errors, defined as number of times the mouse visited non-escape holes (noted as nose pokes into holes), before entering the goal box; and (4) Mean velocity (cm/s) used to determine if performance differences reflected motor ability. If a mouse did not enter the goal box during a 3 minute training trial, 180 seconds was entered as their total latency, and their number of errors and distance travelled during the 3 minute trial were entered as total errors and total distance, respectively. As previous studies have observed that mice may find the goal box but choose to continue exploring the maze (Harrison et al., 2006; Patil et al., 2009), it is recommended to also record latency, distance and

number of errors to the first encounter (nose poke) of the escape hole, called primary latency, primary distance and primary errors respectively, to assess spatial learning. The number of trials where mice did not enter the goal box were also recorded.

Prior to probe trials 1 and 2, the goal box was removed from the escape hole and mice explored the maze for 90 s. No training occurred between the two probe trials. Primary latency, primary distance and primary errors were recorded during the probe trials.

Statistical Analyses

Statistical analyses were performed in R statistical software 4.2.3 (R Core Team, 2020), and figures were produced using GraphPad Prism V9. For comparisons over time, where there were repeated measures (i.e. pup size and spatial learning), models were built with Maternal treatment condition (*H. bakeri* infected versus uninfected), offspring sex (male versus female), and timepoint/trial included as a fixed factors and the identity of the mouse as a random factor (Lazic, 2010). A similar model was built for dam weight. Litter size was included as a covariate when assessing mouse size (pup and dam). Further, as body weight has been shown to negatively influence spatial learning (Cordner and Tamashiro, 2015), and we observed this in our study, offspring weight was included as a covariate in our models for spatial learning. No association was found between body weight and spatial memory performance, thus body weight was not included as a covariate in these models. Non-significant interactions between fixed effects were excluded from models (Zuur et al., 2009). As trends in the anxiety-like behaviour and spatial memory data were observed, where differences were evident between females born to *H. bakeri* infected versus uninfected dams but not between males, data from male and female offspring were analyzed separately. This approach is often taken in the literature (Lante et al., 2007; Batinić et al., 2016); thus, models were built with Maternal treatment condition (*H. bakeri* infected versus uninfected) as the fixed factor.

Extreme outliers were identified in Prism using the ROUT method (a method combining Robust regression and Outlier removal). The strictest cut off of Q = 0.1% was selected to reduce the chance of falsely detecting outliers, meaning only extreme outliers were identified and removed. Extreme outliers occurred only in Probe Trial 1 where two male treatment mice were

outliers for primary latency, distance and errors. Analyses for Probe 1 were performed with these mice included and excluded and exclusion of these outliers did not influence the results.

Using the fisher.test function in R, Fisher's exact tests were used to analyze the number of trials where mice did or did not enter the goal box during the training phase; male and female offspring were analyzed separately. For remaining variables, linear models (LM), negative binomial generalized linear models (NB.GLM), linear mixed models (LMMs) or generalized linear mixed models (GLMMs) were built using the lm, glm.nb, nlme or glmer function, respectively (MASS package (Venables, 2002), nlme package (Pinheiro, 2023) lme4 package (Bates et al., 2015). When necessary, post hoc pairwise comparisons were performed using the emmeans function (emmeans package (Lenth, 2020)) with a Tukey correction. Normality, independence and homogeneity of variances of mixed models were assessed using fitted residuals from the plotresid function (RVAideMemoire package(Hervé, 2020)), and in the case of GLMMs, also using the DHARMa package (Hartig, 2020). Unless otherwise stated, values are presented as means ± SEM. The significance level was set at 0.05.

As no pup mortality occurred, the influence of the maternal infection status on litter size was analyzed on PD 20 using a LM. For dam weight and offspring weight and length, measured over time, LMMs were used with log transformations for weight and length.

Data on number of fecal droppings were discrete and overdispersed and were analysed using NB.GLMs. Variables from the training and probe trials were positively skewed, and in some instances, heteroscedastic. For the training phase, LMMs with log transformations were used for total latency, total distance and mean velocity and Gamma GLMMs, with log link function, were used for primary latency and primary distance. Both total and primary errors were discrete and overdispersed, and negative binomial GLMMs, with log link function, were used. For the probe trials, LMs with log transformations were used for primary latency and primary distance and a NB.GLM was used for primary error.

Results

This study assessed the influence of maternal *H. bakeri* infection on the spatial learning and memory and anxiety-like behaviour of uninfected male and female adult offspring in the BMT. Outbred CD-1 mice were infected repeatedly or sham-infected during pregnancy and lactation and their three-month old adult offspring from 15 uninfected and 16 *H. bakeri* infected dams were used (one male and one female pup per dam). Mortality was consistently zero in this infection model, as expected (Noel et al., 2022).

Impact of Maternal Infection on Dam Mass, Litter Size, and Pup Size

Maternal infection did not influence dam mass at GD 7, 12, or 17 (all P values > 0.05) or litter size (uninfected: 12.2 ± 0.4; infected: 11.8± 0.3; P = 0.44) (Supplementary Fig. 1). As reported in the literature (Kristan, 2002; Odiere et al., 2010; Noel et al., 2022), male and female pups born to infected dams had lower mass and shorter length than pups of uninfected dams at PD 20 (all P values < 0.001, Supplementary Fig. 2). For the first time, it was observed that this impaired growth persisted in adult offspring at PD 69 (all P values < 0.001, Supplementary Fig. 2). As expected, mass and length were greater in males than females (Kristan, 2002; Noel et al., 2022) at both PD 20 and PD 69 (all P values < 0.01, Supplementary Fig. 2).

Impact of Maternal Infection on Offspring Anxiety-like Behavior and Spatial Learning and Memory in the Barnes Maze Test

Anxiety-like behaviour during habituation trial

Defecation is a sign of fear or anxiety in rodents, which is often observed when they are placed in a novel environment (Denenberg, 1969; Bailey and Crawley, 2011). Thus, the number of fecal pellets were counted during the five min habituation trial when mice were introduced to the maze, as a measure of anxiety-like behavior. Female offspring of *H. bakeri* infected mothers had significantly fewer fecal pellets than female offspring of uninfected mothers (P = 0.02; Fig. 2), suggesting that maternal infection during pregnancy and lactation reduced anxiety in their uninfected adult female offspring. This difference was not observed in the males.

Spatial learning and anxiety-like behaviour in the training phase

As mice may continue to explore the maze after finding the goal box, two sets of spatial learning variables were explored, those related to first arriving at the escape hole which contained the goal box ("primary" variables), and those related to entering the goal box ("total" variables). Primary variables were a better indication of spatial learning whereas total variables were used to assess spatial exploration as an indicator of anxiety-like behaviour.

Spatial learning: Regardless of maternal infection or offspring sex, adult offspring learned the location of the escape hole, based on the first nose poke into the escape hole, on the first training day as indicated by a decrease in the average primary latency ($P < 0.0001$; Fig. 3a), primary path length ($P < 0.0001$; Fig. 3b), and primary errors ($P < 0.0001$; Fig. 3c) between training days 1 and 2. Thereafter, values remained low. Neither maternal infection nor offspring sex influenced mean velocity (Fig. 3d), indicating no differences in motor ability. Of note, independent of maternal infection, primary latency and primary distance were negatively associated with offspring weight (all P values < 0.05, data not shown).

Anxiety-like behaviour: Adult female offspring of infected mothers had higher total latency ($P = 0.044$; Fig. 4a), total distance ($P = 0.058$; Fig. 4b) and total errors ($P = 0.043$; Fig. 4c) compared to female offspring of uninfected mothers. No differences were observed between adult male offspring of uninfected and infected mothers in total latency, total distance and total errors (Supplementary Fig. 3). Furthermore, although all mice found the goal box during each training trial, a significantly higher percentage of females of infected dams did not enter the goal box (17.2%) compared with females of uninfected dams (4.6%) ($P<0.0001$). There was no difference in the percentage of training trials where males did not enter the goal box (males of infected mothers: 6.6%; males of uninfected mothers: 4.6%; $p = 0.34$). Taken together, these data indicate that females of infected mothers were less anxious as they were less motivated to seek shelter in the goal box during training trials, and more inclined to explore the maze after finding the goal box.

Probe trials of spatial memory

During probe trial 1, which assessed short-term spatial reference memory, maternal infection did not influence the time taken (primary latency) or distance travelled (primary distance) to find the escape hole for adult female offspring (Fig. 5a-b). However, adult female offspring of infected mothers made half as many primary errors before first finding the escape hole compared to offspring of uninfected mothers ($P = 0.043$; Fig. 5c) indicating better short-term spatial reference memory in response to maternal *H. bakeri* infection. These differences were not detected in the adult male offspring (Fig. 5a-c).

During probe trial 2, which assessed long-term spatial reference memory, females of infected mothers travelled a shorter primary distance ($P = 0.049$; Fig. 5b) and although not significant, they appeared to make fewer primary errors ($P = 0.073$; Fig. 5c) in finding the escape hole compared to females of uninfected mothers. These results provide evidence of better long-term spatial reference memory in adult female offspring of infected dams. There were no differences in long-term spatial reference memory between males of uninfected or infected mothers (all P values in probe 1 and 2 > 0.05; Fig. 5a-c). The fewer primary errors in probe trial 1 and shorter primary distance in probe trial 2 provide evidence that maternal infection may enhance spatial memory in adult female mice.

Discussion

Using a GI nematode parasite that remains in the maternal intestine, the main goal of this study was to assess if the positive influence of maternal infection during pregnancy and lactation on spatial memory of juvenile male and female offspring was retained in the uninfected adult offspring. Some indicators of anxiety-like behavior in adult offspring were also explored. It was reported for the first time that female adult offspring of *H. bakeri* infected dams retained enhanced spatial reference memory and also exhibited signs of reduced anxiety-like behaviour compared to females of uninfected dams. No differences were observed in the behaviour of adult male offspring of infected vs. uninfected dams. It is hypothesized that sex hormones may at least in part explain the sex-specific differences in behavioural responses to maternal infection of adult offspring.

Previous findings showed that maternal *H. bakeri* infection enhanced spatial memory in both juvenile male and female offspring, where PD 17 pups of infected dams retained object location memories for three hours in the Object Location Test but offspring of uninfected mothers did not, and where PD 34 juveniles of infected mothers retained their ability to find an escape location in the BMT for one week but offspring from uninfected mothers did not (Noel et al., 2022). These findings were consistent with the enhanced hippocampal LTP in uninfected three-week old offspring of *H. bakeri* infected dams as evidenced by both gene expression and electrophysiological data, and the up-regulated neurogenesis pathway (Noel et al., 2024), all of which are strongly associated with spatial memory (Abrous and Wojtowicz, 2015; Lieberwirth et al., 2016). Further, maternal *H. bakeri* infection also up-regulated expression of brain derived neurotrophic factor (BDNF) in the whole brain of neonates (unpublished data from our lab; $p = 6.8E-05$), and hippocampus of juveniles (Noel et al., 2024). BDNF is a key molecule for learning and memory as it is involved in neurogenesis and synaptic plasticity (Lu et al., 2008; Miranda et al., 2019), and it also plays an important role in reducing behaviours associated with anxiety (Tatiana Marins et al., 2020; Yin et al., 2022). It has been suggested that these differences may be due to the Th2/Treg biased neuroimmune environment observed in the hippocampus of uninfected male and female juvenile offspring of *H. bakeri* infected mothers (Noel et al., 2024), a neuroimmune environment that mimics the systemic immune response of the infected mother (Odiere et al., 2013; Su et al., 2023).

The present study has shown that the enhanced spatial reference memory observed in juvenile offspring in response to maternal *H. bakeri* infection (Noel et al., 2022) was retained in adult female offspring. This was evidenced by adult female offspring of infected mothers making half the number of errors before finding the escape hole in both probe trials, and having a more direct path to find the escape hole in probe trial 2 in comparison to adult females of uninfected mothers. These differences were not observed between male offspring of infected vs. uninfected mothers. The present study also provides evidence of decreased anxiety-like behaviour in adult female offspring of *H. bakeri* infected mothers, as they produced fewer fecal pellets in the 5 min habituation trial, compared with the adult female offspring of uninfected mothers. In addition, during the training phase, despite finding the goal box which offered protection from the brightly lit and exposed maze, adult female offspring of infected dams chose to continue exploring instead of seeking shelter and thus did not enter the goal box as soon as females of uninfected

mothers. Again, these differences were not observed between male offspring of infected vs. uninfected mothers. Previous studies have found that maternal physical exercise enhances offspring spatial memory and decreases anxiety-like behaviour in three-week old juveniles and four-month old adult rats of both sexes (Aksu et al., 2012; Dayi et al., 2012) and that this is associated with an increase in hippocampal BDNF and neurogenesis (Bick-Sander et al., 2006; Lee et al., 2006; Kim et al., 2007; Aksu et al., 2012; Dayi et al., 2012; Robinson and Bucci, 2012; Akhavan et al., 2013; Gomes da Silva et al., 2016). Considering that juvenile male and female offspring of infected mothers have enhanced hippocampal LTP and up-regulated genes associated with BDNF and neurogenesis (Noel et al., 2024), it is hypothesized that heightened hippocampal BDNF, neurogenesis and LTP may persist in adult females of *H. bakeri* infected dams, driving at least in part the enhanced spatial memory and decreased anxiety-like behaviour that was observed.

It is of considerable interest that the enhanced spatial memory in response to maternal *H. bakeri* infection persisted into adulthood in female but not male offspring. Previous maternal infection models, which have primarily used bacterial or viral infections, or their mimics, have observed sex-dependent effects of maternal infection on the behaviour of mouse offspring (Bergdolt and Dunaevsky, 2019). Unlike an *H. bakeri* infection which regulates the host immune system, limits pathology, and can be considered "anti-inflammatory" in nature (Maizels et al., 2012; Reynolds et al., 2012), bacterial and viral infections are associated with inflammation and harmful pathology in their host (Britton and Saunders, 2010; Rouse and Sehrawat, 2010). In these maternal bacterial and viral models, the strong proinflammatory Th1 immune response in the infected mother is mimicked in the offspring brain and is associated with impaired hippocampal LTP and spatial memory (Boksa, 2010; Bergdolt and Dunaevsky, 2019). In response to maternal LPS or PolyI:C, male offspring display significant spatial learning and memory impairments, whereas females do not (Lante et al., 2007; Howland et al., 2012; Wischhof et al., 2015; Batinić et al., 2016; Gogos et al., 2020). The ability of the female sex hormones, estrogen and progesterone, to dampen Th1 immunity (Roved et al., 2017) may offer protection to female offspring in these models, by ameliorating the damaging neuroinflammatory responses. In contrast to LPS and Polyl:C models, maternal *H. bakeri* infection induces a Th2/Treg, not a Th1, immune response in the mother (Odiere et al., 2013; Su et al., 2023), which is mimicked in the brain of neonatal and juvenile offspring and is suggested

to promote hippocampal BDNF, neurogenesis, LTP and spatial memory (Haque et al., 2019; El Ahdab et al., 2021; Noel et al., 2024). Given that estrogen and progesterone enhance Th2 immune responses (Roved et al., 2017), it is possible that the influence of these sex hormones on the immune system may allow the Th2/Treg biased neuroimmune environment to persist in adult female offspring of *H. bakeri* dams, explaining the positive influence on female behaviour at adulthood.

In contrast to estrogen and progesterone, the male sex hormone, testosterone, dampens Th2 immune responses (Hepworth et al., 2010; Klein and Flanagan, 2016; Roved et al., 2017; Taneja, 2018). Evidence of this is seen in mice infected with *H. bakeri* (Dobson, 1961; Van Zandt et al., 1973; Prowse et al., 1979; Maizels et al., 2012; Rynkiewicz et al., 2019) or the GI nematode *Trichuris muris* (Hepworth et al., 2010), where male mice are less resistant and harbour more worms than female mice (Dobson, 1961; Van Zandt et al., 1973; Prowse et al., 1979; Maizels et al., 2012; Rynkiewicz et al., 2019), due to the suppression of protective Th2 immunity by testosterone in males (Hepworth et al., 2010; Roved et al., 2017). It is thus possible that the Th2/Treg biased neuroimmune environment observed in juvenile male and female offspring of *H. bakeri* infected dams (Noel et al., 2024), which was hypothesized to promote spatial memory (Noel et al., 2022; Noel et al., 2024), may be retained in adult female offspring but dampened by testosterone in adult males, explaining why enhanced spatial memory in adult male offspring was no longer observed. Given that circulating sex hormones are at low levels in prepubescent mice (Bell, 2018), they would not have been expected to have a strong influence on the immune response in juvenile offspring.

The ecological effects of a GI nematode infection that enhances spatial memory and reduces anxiety-like behaviour of female offspring are unknown. Spatial memory in mice is necessary for mate location, foraging, predator avoidance and territorial defence, and it is therefore an essential aspect of survival (Vorhees and Williams, 2014). Enhancement of spatial memory might thus be beneficial. Additionally, the reduced anxiety-like behaviour might complement the enhanced spatial memory, as adult female offspring of *H. bakeri* infected dams would be less anxious and more inclined to explore, increasing opportunities for foraging. Of note, however, reduced anxiety-like behaviour could be a disadvantage with respect to safety and predator avoidance. Lastly, given that reproductive effort and parental investment are more

costly for female mice than for males (Parmigiani et al., 1994), there could be evolutionary advantages if females have better spatial memory and lower anxiety.

The following limitations are acknowledged. First, the estrous cycle of adult female offspring was not controlled for. Although estrous cycle is recognized as a strong determinant in emotionality and cognitive capacity of female rodents (Gawel et al., 2019), the large sample size, and use of the BMT which spanned 13 days (i.e. three estrous cycles/ female mouse (Byers et al., 2012)), makes it likely that all the phases were represented. Second, a test designed to specifically assess anxiety behaviour in mice (e.g. elevated plus maze) would have provided more conclusive data on the influence of maternal *H. bakeri* infection on anxiety behaviour in offspring. Third, we were unable to determine whether the neuroimmune environment of adult female offspring of infected dams was altered in response to maternal *H. bakeri* infection, as it had been in the juvenile offspring of infected dams (Noel et al., 2024).

To the best of our knowledge, this is the first study to assess the impact of a maternal GI helminth infection on the spatial memory of adult offspring and to determine if the positive influence observed in juvenile male and female offspring persisted into adulthood. It was observed that maternal GI nematode infection during pregnancy and lactation enhanced spatial memory and may also reduce anxiety-like behavior in adult female, but not male, offspring. It will be of great interest to determine whether sex hormones are the driving factor behind these observations, if this maternal infection influences other aspects of offspring behaviour, and if other nematode infections such as *Nippostrongylus brasiliensis,* also alter offspring behaviour.

Data availability

The datasets generated and analysed for this study are available via a link to the Borealis Dataverse [https://doi.org/10.5683/SP3/HVCHM4], a public data repository.

Author contributions

S.C.N. conceived and designed the study, conducted experimental work, analyzed the data, interpreted the results, and drafted the manuscript. R.L. assisted with experimental work and extraction of data from the behavior videos using the Ethovision XT software. M.E.S.

provided input on the study design and data interpretation, as well as critical suggestions that have been incorporated into the manuscript, and obtained funding for the research.

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Competing Interests

The authors declare no competing interests.

Ethical Standards

This study (protocol #2000– 4601) was approved by the McGill University Animal Care Committee according to the guidelines of the Canadian Council on Animal Care. All methods were carried out in accordance with relevant guidelines and regulations, and the study was carried out in compliance with ARRIVE guidelines (https://arriveguidelines.org).

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Figures

Figure 1. Schematic representing experimental design and protocol. Of the 40 timed-pregnant dams received on gestation day (GD) 4, only 31 delivered litters. On postnatal day (PD) 20, one pup per sex per litter was selected to perform the Barnes Maze Test. Of the pups selected for behavioural analysis, their size, specifically crown-rump length and weight, were recorded on PD 20 and 69 (see Supplementary Figure 2).

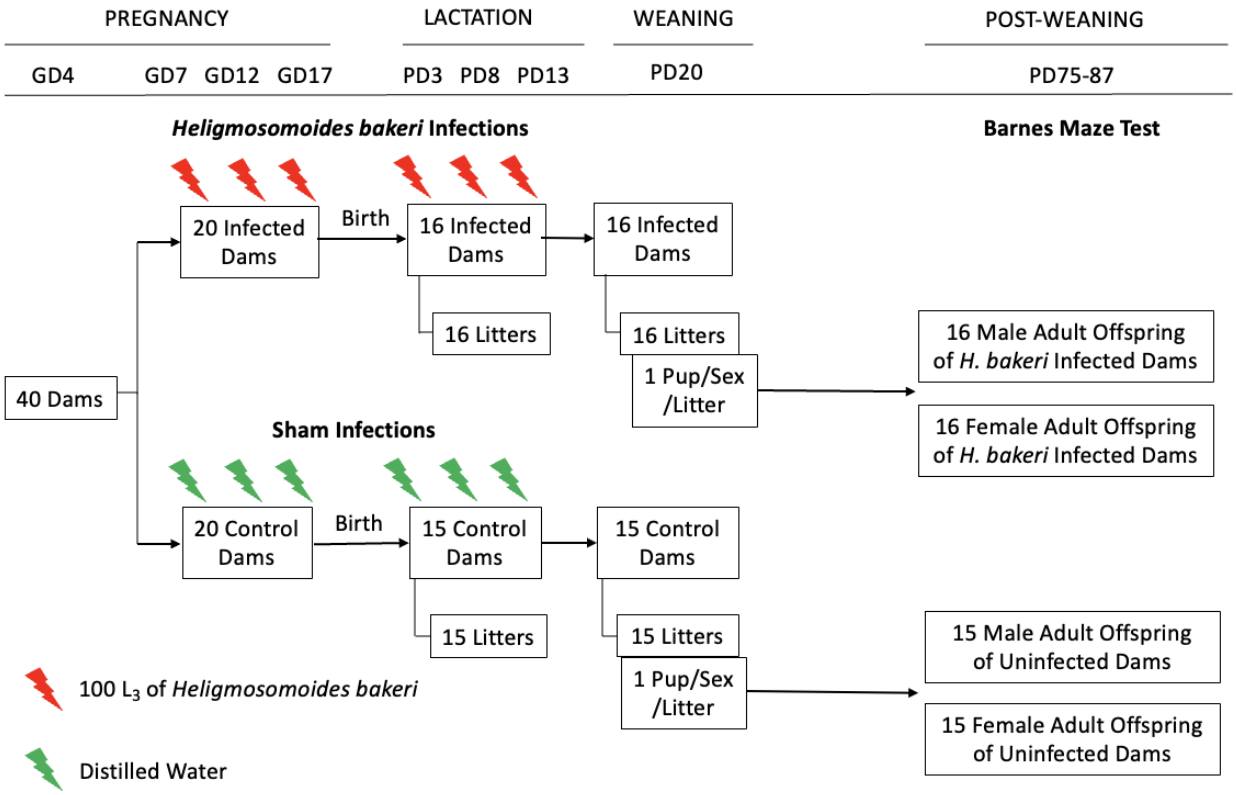


Figure 2. Maternal *H. bakeri* infection reduced female offspring fecal count during the five minute habituation trial of the Barnes Maze Test. Values are means±SEM, n = 15-16 offspring per group (*P < 0.05; ns = not significant).

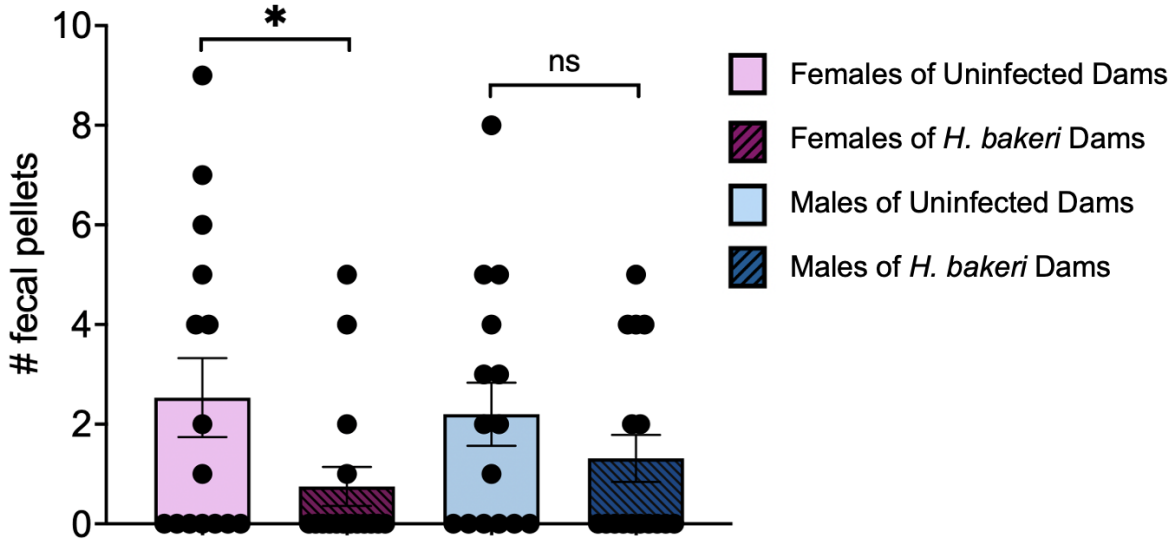


Figure 3. Neither maternal *H. bakeri* infection nor adult offspring sex influenced primary variables of spatial learning in the Barnes Maze Test over four training days. All mice performed significantly better after the first training day. Values are LSmeans±SEM, n = 15-16 offspring per group. **(a)** primary latency, **(b)** primary distance and **(c)** number of primary errors to reach the escape hole, and **(d)** mean velocity during the trial. Different letters show the effect of training day, $P < 0.05$.

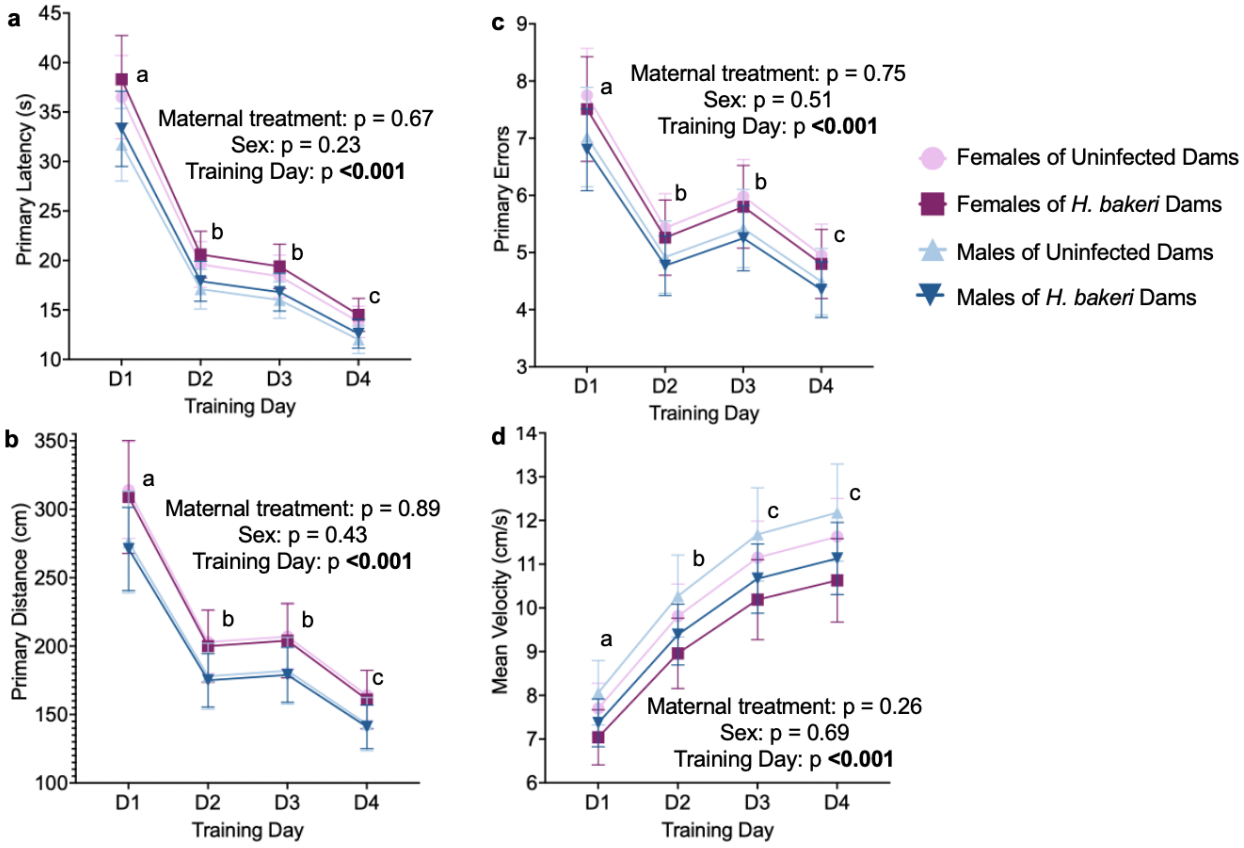


Figure 4. Maternal *H. bakeri* infection influenced female offspring exploration during the four day training phase of the Barnes Maze Test. Total parameters were used as an indication of exploration to provide an understanding of fear/anxiety levels. Values are LSmeans±SEM, n = 15-16 offspring per group. **(a)** total latency, **(b)** total distance and **(c)** number of total errors to enter the goal box.

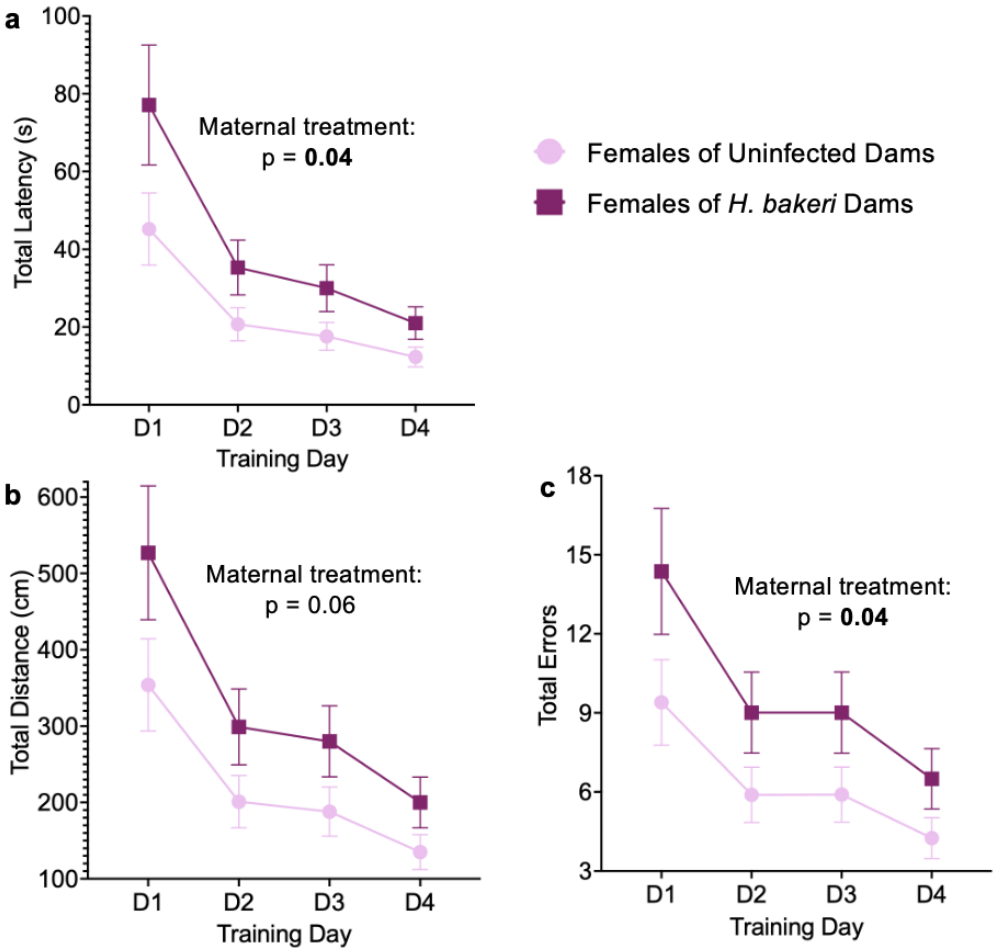
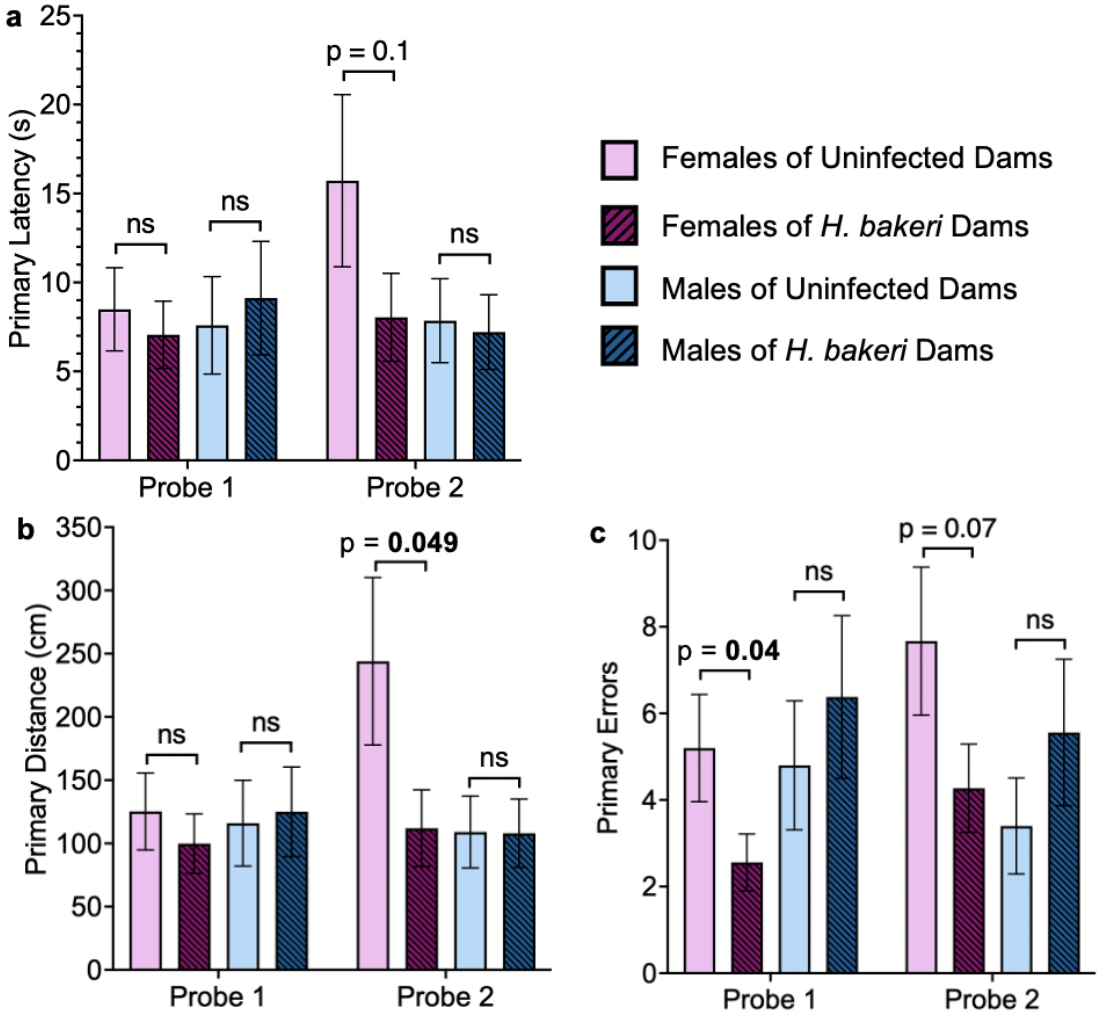
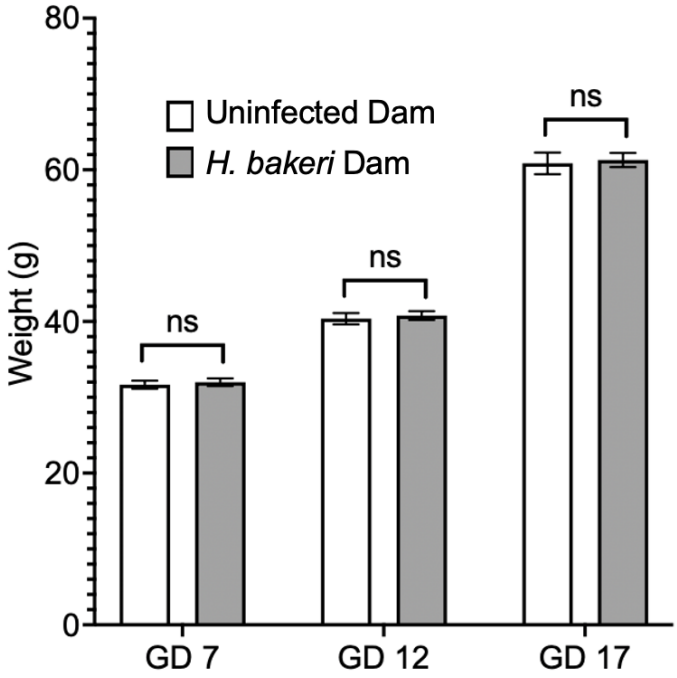


Figure 5. Maternal *H. bakeri* infection influenced adult female offspring short-term (probe trial 1) and long-term (probe trial 2) spatial reference memory in the Barnes Maze Test but not male offspring spatial reference memory. Probe trial 1 was conducted 24 hours after the last training day and probe trial 2 was conducted one-week later. Values are means±SEM, n = 14-16 offspring per group (ns = not significant). **(a)** primary latency, **(b)** primary distance and **(c)** number of primary errors to reach the escape hole.

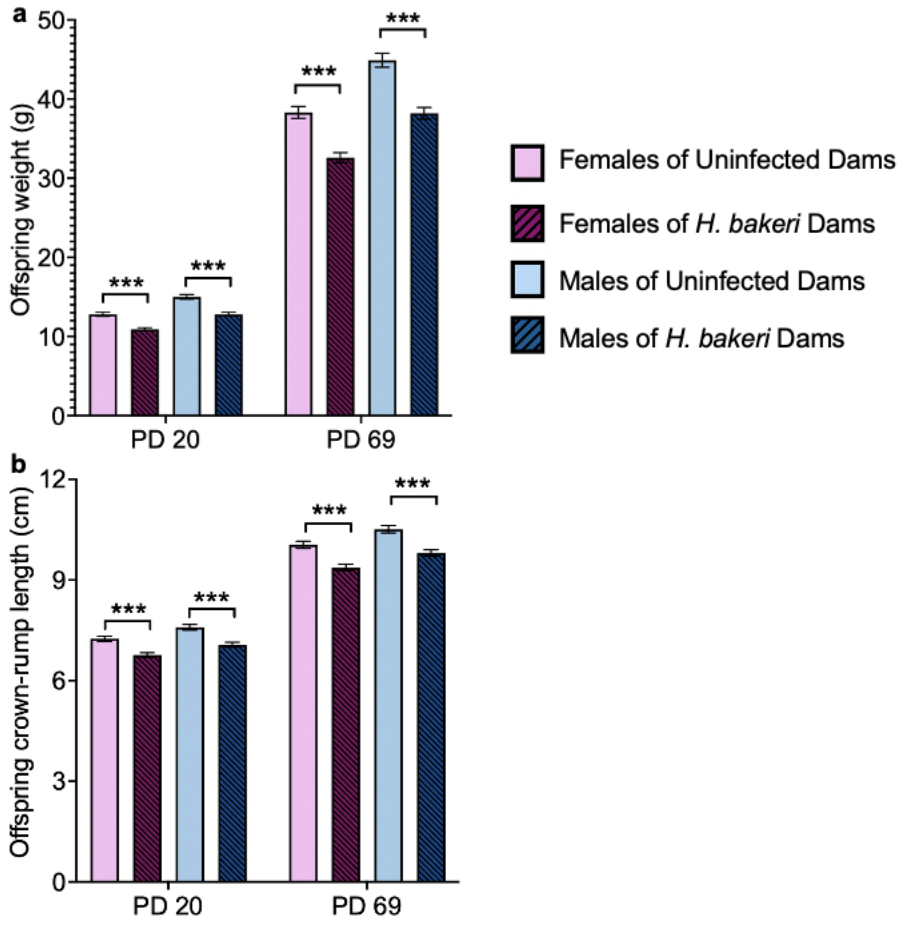


Supplementary Figures

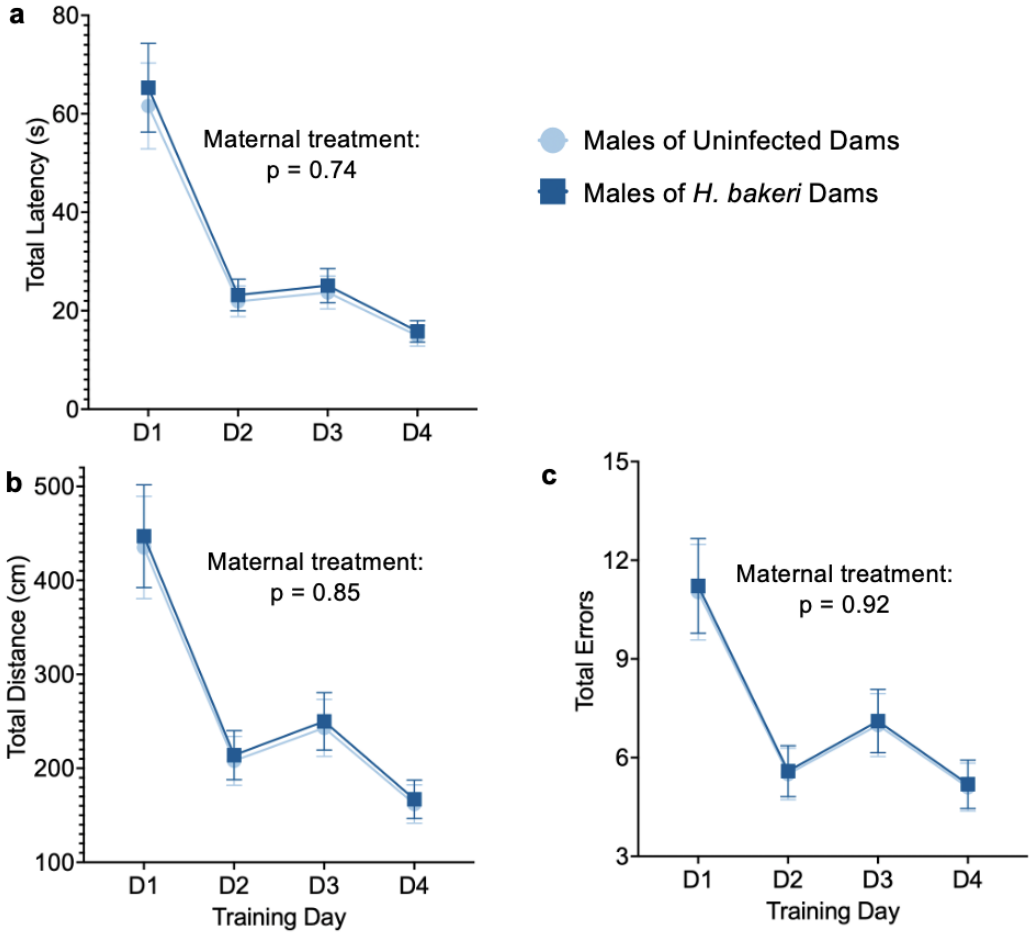
Supplementary Figure 1. Maternal *H. bakeri* infection did not influence dam weight at gestation day (GD) 7, 12 and 17. Litter size was included as a covariate. Values are means±SEM, n = 15-16 per group (ns = not significant).



Supplementary Figure 2. Maternal *H. bakeri* infection influenced offspring size at postnatal day (PD) 20 and 69. Litter size was included as a covariate. Values are means±SEM, n = 15-16 offspring per group (***$P < 0.001$). **(a)** Offspring body mass and **(b)** offspring crown-rump length.



Supplementary Figure 3. Maternal *H. bakeri* infection did not influence male offspring exploration during the four day training phase of the Barnes Maze Test. Total parameters were used as an indication of exploration to provide an understanding of fear/anxiety levels. Values are LSmeans±SEM, n = 15-16 offspring per group. **(a)** total latency, **(b)** total distance and **(c)** number of total errors to enter the goal box.



Chapter VI - General Discussion

6.1. Maternal *H. bakeri* infection influenced offspring hippocampal neuroimmunity, LTP and spatial memory and improved resistance to direct infection

Before starting my PhD, it was known that *H. bakeri* infection during pregnancy and lactation increased circulating Th2 cytokines (IL-4, IL-5, IL-10 and IL-13) in the infected dam[8,9], and that parasite-specific IgG1[9] was transferred (via nursing) to pre-weaned 10-day old neonates, protecting them against *H. bakeri* [7]. It was also known that fetal and juvenile (PD 14 and 21) offspring of *H. bakeri* infected dams had lower crown-rump length[18-20], and that juvenile offspring also had lower body mass[20], but the impact on growth in adult offspring had not been explored. Further, it was shown that maternal *H. bakeri* infection altered placental gene expression [158], as well as brain gene expression of the developing fetus[162] and uninfected seven-day old male neonate[3]. Of great interest, in response to maternal *H. bakeri* infection, the neonatal brain gene expression showed up-regulation of five key interacting pathways associated with LTP [3], and suggested this may be driven by a Th2/Treg biased neuroimmune environment[3,10]. As LTP of glutamatergic synapses is a form of activity-dependent synaptic plasticity that is a leading candidate for the neural substrate underlying learning and memory[4,65,274,276], this finding suggested that maternal GI nematode infection during pregnancy and lactation may actually be beneficial to the cognitive function of offspring. However, the phenotypic implications of the altered brain gene expression in response to maternal *H. bakeri* infection was not tested. As such, the possible consequences of maternal GI nematode infection on the cognitive function of uninfected offspring were largely unknown when I began my PhD.

In humans, two studies had assessed the influence of maternal GI nematode infection on offspring cognition, with one study finding a negative association between maternal hookworm infection and infant cognition[37], and the other study finding no association[38]. Of note, the study which found a negative influence of maternal hookworm infection on infant cognition did not assess iron deficiency anemia (e.g. hemoglobin levels, blood iron levels or ferritin levels) in the mother or infant[37], despite iron deficiency being known to have strong negative impacts on

hippocampal development and offspring cognition[121-127]. Given that there is a large research gap associated with the influence of maternal GI nematode infection on offspring cognitive function, given that one-third of all pregnant women in endemic settings are estimated to be infected with hookworm[34], and given efforts are currently underway to utilize GI nematodes for the treatment of autoimmune diseases and IBD in humans[79,80], this dissertation was designed to explore the possibility that maternal GI nematode infection may influence the cognitive function of uninfected offspring, with a specific focus on spatial memory.

The key findings are that maternal GI nematode infection enhanced spatial memory of two and three-week old juvenile male and female offspring and this was retained in adult three-month female offspring. With regards to the three-week old juvenile offspring of infected mothers, my evidence suggests that enhanced spatial memory was due to enhanced hippocampal LTP, evidenced by my electrophysiological and gene expression data, and that this may be the result of accelerated maturation of glutamatergic synapses. In addition, as neurogenesis is positively associated with spatial memory performance[16], and early development of hippocampal myelination promotes excitatory synaptic transmission and cognitive function[17], evidence from my gene expression analysis suggests the enhanced LTP and spatial memory in juvenile offspring may also be associated with the up-regulation of genes associated with neurogenesis and myelination in the hippocampus of offspring in response to maternal infection. Further, I observed transfer of protective immunity from the *H. bakeri* infected mother, as evidenced by a greater resistance to *H. bakeri* infection in their two-month old offspring, consistent with maternal transfer of parasite-specific IgG1, which was evident in the serum of PD 24 uninfected offspring. My data from juvenile offspring suggests the transfer of immunity from the *H. bakeri* infected mother extends to the offspring hippocampus, as the immunoregulatory TGF-β signaling pathway was up-regulated, a greater number of microglia and astrocytes were observed, and a higher percentage of CD206 positive microglia which are typically increased in response to the Th2 cytokine, IL-4 [11-13,277], was also found. These findings revealed a potential mechanism behind the enhanced LTP and spatial memory, as both TGF- β and IL-4 are known to have measurable downstream effects on LTP and spatial memory[22,26,278-280]. Finally, I also observed for the first time that the influence of maternal *H. bakeri* infection on offspring growth extends into adulthood, as both male and female adult offspring had lower crown-rump length and body mass compared to offspring of uninfected dams. Taken together,

my findings show for the first time that maternal GI nematode infection positively influences the spatial memory of uninfected offspring, possibly through transfer of a Th2/Treg immune phenotype from the infected dam that protects the offspring from direct infection and extends to their developing brain.

6.2. Possible pathways by which maternal *H. bakeri* infection promotes offspring spatial memory and future directions

The benefit to offspring spatial memory was intriguing as the nematode infection remained restricted to the maternal gastrointestinal tract and thus offspring were uninfected. The following potential pathways could explain how cues of a maternal *H. bakeri* infection might have interacted with the developing brain to promote spatial memory.

6.2.1. Maternal transfer of immune elements

First, maternal transfer of immunity (i.e. antibodies, cytokines and immune cells) both *in utero* and via nursing can shape the offspring immune system[7,35,36,101,281], which in turn can influence brain development and function[6]. With respect to *H. bakeri* infected dams, prior studies have observed an increase in circulating Th2 cytokines (IL-4, IL-5, IL-10 and IL-13) and *H. bakeri* -specific IgG1 and a decrease in Th1 (IFN-γ) and Th17 (IL-17) cytokines. Further, it was previously found that *H. bakeri*-specific IgG1 was transferred, via nursing, to pre-weaned 10-day old neonates, which protected them against *H. bakeri* [7]. Similarly, I observed that weaned, 24 day old uninfected offspring, had detectable levels of *H. bakeri*-specific IgG1 in their serum, which was consistent with their enhanced resistance to direct *H. bakeri* infection in comparison to offspring of uninfected dams. Although this was previously observed in neonatal offspring[7], this is the first study to observe enhanced resistance and maternal transfer of *H. bakeri*-specific IgG1 in juvenile weaned offspring of *H. bakeri* infected dams. Whether this resistance to infection extends to adulthood, is unknown. My findings indicate that immune molecules are transferred from the infected dam to their uninfected offspring, which in addition to protecting them from early-life infection, may also influence their neuroimmune system, and in turn their neurodevelopment and cognitive function.

The exact mechanism(s) by which immune molecules and/or cells that are transferred from the infected mother to their offspring can influence the neuroimmune system, and in turn neurodevelopment and cognitive function, is unknown. However, there are a number of known pathways by which peripherally-derived immune factors can affect the brain, including the travelling or signalling of cytokines, chemokines, and leukocytes across the blood brain barrier (BBB) [206]. A previous study from our lab suggested that maternal *H. bakeri* infection may promote the transport of immune elements across the BBB, as brain gene expression data from seven-day old neonates of *H. bakeri* dams indicated up-regulation of leukocyte transendothelial migration, endocytosis, T and B cell receptor signaling and Th2/Treg pathways, and genes for IL-4 and TGF-β[3,10]. Consistent with this, in the hippocampus of three-week old offspring, I also observed up-regulation of the leukocyte transendothelial migration, endocytosis and TGF-β signaling pathway in response to maternal *H. bakeri* infection. Considering that endocytosis[282,283] and leukocyte transendothelial migration[284,285] allow immune elements such as antigens, immunoglobulins, cytokines, and leukocytes to cross the BBB, this may explain how immune signals that are transferred from the *H. bakeri* infected mother might reach the offspring brain. The ways in which immune elements transferred from the *H. bakeri* infected dam might influence spatial memory of the uninfected offspring are detailed below.

The Th2 cytokines, IL-4 and IL-13, have been previously shown to be vital for spatial memory formation, as they stimulate astrocytes and microglia to produce BDNF, a key molecule for LTP and cognitive function[286], and this drives spatial memory formation[12,22,27,210,280]. In addition, TGF-β signaling is critical for differentiation, development and function of neurons and glia, and is vital for neurogenesis and myelination[287,288], both of which are positively associated with spatial memory[16,17]. Thus, if Th2/Treg cytokines are transferred from the infected mother to the offspring and either travel or signal across the BBB to influence glial cells, this could promote spatial memory. My study provides evidence of this. Firstly, I observed an increase in the number of microglia and astrocytes in the hippocampus of offspring in response to maternal infection, indicating altered neuroimmunity. Although an increase in the density of microglia and astrocytes can be triggered by Th1 cytokines which drive reactive phenotypes and neuroinflammation[11,43], my data indicated a phenotype that was responding to Th2/Treg cytokines and may have pro-cognitive functions. Specifically, I observed an up-regulation of genes encoding TGF-β2 and its receptor TGF-βR1, and a greater percentage of microglia with

the CD206 marker, in the hippocampus of offspring of *H. bakeri*-infected dams, all of which are associated with a microglia phenotype responding to Th2 cytokines[11-13,270]. In addition, I observed that *Serpina1* and *Sphk1* genes, which are normally up-regulated in reactive astrocytes in response Th1 cytokines[271,289], were significantly down-regulated in my gene expression data. Further, in response to Th2/Treg cytokines, astrocytes up-regulate *Aqp4, Tgfb, CD109, Ptx3, Nfia, Bdnf,* and *Gdnf*, and have neuroprotective and pro-cognitive properties[271,290-292], and these genes were all up-regulated in response to maternal *H. bakeri* infection. I also observed that the TGF-β signaling pathway was up-regulated in response to maternal infection, as was the expression of 13 key genes associated with oligodendrocytes and myelination (*Olig1*, *Olig2*, *Sox10*, *Nkx2.2, Myrf, Zfp488, Cldn11*, *Plp1, Foxo4* , *Cnp*, *Mbp, Mag and Mog*). Taken together, I hypothesize that maternal infection may promote the signalling and/or transport of Th2/Treg cytokines across the BBB which influences the development and function of glial cells in a direction that promotes spatial memory.

In addition to cytokines, it is also important to consider that during pregnancy, maternal cells are transferred to the fetus, where they can reach the developing brain[293]. Maternal cells which are vertically transferred to the fetus during mammalian pregnancy occur in low numbers in the offspring, and these cells have been termed maternal microchimeric cells (MMc)[293]. The transfer of MMc from mother to fetus commences with maturing placentation, which occurs during mid-gestation in mice[294]. Interestingly, MMc are not rejected by the fetal immune system and can persist long-term in offspring's organs, including their brain [293,295]. Evidence also indicates that MMc can be transferred to the offspring's brain postnatally via breastmilk[296]. Although MMc decrease with increasing age, a recent study observed that MMc were still detectable at low numbers in offspring's brain at PD 60 [293]. Although the influence of MMc on offspring brain function is still largely unknown, it was recently shown that MMc promote microglia homeostasis, early brain wiring and cognitive development of mice[293]. It is unknown how a maternal *H. bakeri* infection might influence the transfer of MMc to the uninfected offspring. However, it has been shown that Th2-competent CD4+ T cells are transferred during nursing from *N. brasiliensis* infected mothers to their offspring, and that these cells are still present and functional in adult offspring[101]. As it is known that CD4+ Th2 cell derived IL-4 and IL-13 is vital for spatial learning and memory[22,27], and that these cells are increased in response to an *H. bakeri* infection[64], it is possible that these cells are transferred

from the *H. bakeri* infected mother to the uninfected offspring, which promotes their spatial memory. Further, as these cells may survive long-term in the offspring, it is possible this may explain how the enhanced spatial memory observed in juvenile offspring is maintained into adulthood in females of *H. bakeri* dams.

In addition, the high levels of parasite-specific IgG1 in the serum of uninfected offspring of infected dams may also play a role, as systemic administration of IgG has been observed to dampen neuroinflammation, and improve memory deficits in a mouse model of Alzheimer's[297]. Also, in a mouse model of traumatic brain injury, intravenous administration of IgG was shown to reduce microglia activation and proinflammatory cytokine production, and enhance neurogenesis and spatial memory[298]. Despite the mechanism being unknown, IgG can cross the BBB[299], thus, it is possible the transfer of high levels of parasite-specific IgG1 from the infected mother to the serum of uninfected offspring may travel or signal across the BBB to influence neuroimmunity and promote spatial memory.

Finally, during normal brain development, gene expression is regulated by a pattern of epigenetic changes including histone modifications, DNA methylation, and microRNA (miRNA) expression[43]. These epigenetic mechanisms also play a role in spatial memory[300-304] and immune responses[305]. Of interest, there is increasing evidence that maternal factors, such as viral infections[306,307] and stress[308], may influence epigenetic mechanisms in the offspring brain that underlie disruption to gene expression, mediating cognitive impairments. It is unknown whether a maternal GI nematode infection influences epigenetic mechanisms in the offspring brain. However, *H. bakeri* is known to release exosomes which contain miRNAs that regulate host genes to elicit immunomodulatory effects and facilitate parasite survival[309,310]. Further, microarray analysis of mouse cells incubated with *H. bakeri* exosomes *in vitro* identified *Dusp1* as the most strongly down-regulated gene, and showed *Dusp1* can be repressed by *H. bakeri* miRNAs based on a reporter assay[310]. This study highlighted that reduced *Dusp1* levels may influence the host immune system and would likely favour parasite survival[310]. This finding is of great interest as my gene expression data shows the *Dusp1* gene was significantly down-regulated in the hippocampus of offspring in response to maternal *H. bakeri* infection. This indicates that parasite-derived exosomes might be transferred from the infected mother to the offspring brain, which may influence their brain gene expression and underlie changes to the neuroimmune system which promote spatial memory.

I have highlighted a few important questions to answer for follow up studies. 1) Does maternal *H. bakeri* infection influence BBB integrity and is there evidence of increased transport of cytokines or leukocytes across the BBB? 2) Does maternal *H. bakeri* infection influence transfer of MMc? More specifically, is there evidence of transfer of CD4+ Th2 cells from the infected mother to the uninfected offspring serum or brain? 3) Does maternal *H. bakeri* infection increase IL-4, IL-13, TGF-β, and BDNF protein levels in the hippocampus of offspring? 4) Does maternal *H. bakeri* infection increase levels of IgG1 in the brains of uninfected offspring? Also, does transfer of *H. bakeri*-specific IgG1 to uninfected offspring of uninfected dams enhance their spatial memory? 5) Does maternal *H. bakeri* infection influence microglia and astrocyte function? Although my work suggests this maternal infection is influencing the function of these cells, further experiments are needed. As was performed by Mattei et al.[58], it would be ideal to isolate these cells from the hippocampus and measure cytokine and BDNF mRNA levels. It would also be of interest to utilize techniques like single-cell RNAseq. 6) Is there evidence of *H. bakeri* exosomes in pup circulation and are *H. bakeri*-derived miRNAs present in the brain of offspring born to *H. bakeri* infected mothers?

6.2.2. Transfer of maternal microbiome

It has been observed that maternal *H. bakeri* infection alters the maternal gut microbiome as well as the gut microbiome of uninfected neonates[105] and three-month old offspring[9], and the microbiome is another means by which brain development and function can be influenced[311,312].

The acquisition of microbes occurs primarily at birth, with delivery through the birth canal exposing the neonate to its mother's microbiome, resulting in vertical transmission of an initial maternal signature[104]. After birth, several factors influence the microbiome composition in early life, including breastfeeding, nutrition, infection, antibiotic use, environmental stressors, and host genetics[313]. It is understood that the gut microbiome plays a fundamental role in the development and function of the host immune system[314]. The gut microbiome also plays a role in neurodevelopmental processes such as the formation of the BBB, myelination, neurogenesis, and microglia maturation, and modulates many aspects of animal behavior, including

cognition[312]. Thus, the gut microbiome can be highly influential with regards to the overall health of the host.

Dysregulation of the gut microbiome has been correlated with a number of adverse consequences, including impaired BBB integrity[315], immune dysfunction and neuroinflammation [316,317], faulty neuronal circuits[318], a defective hypothalamic-pituitary-adrenal axis (HPA) axis [319], and lasting behavioral abnormalities[320]. Maternal immune activation (MIA) (i.e. administration of inflammatory stimuli, such as viral or bacterial pathogens or their mimics, during pregnancy) results in GI barrier defects, abnormal intestinal cytokine profiles, dysbiosis of gut microbiota, altered serum metabolomic profile, neuroinflammation and behavioural abnormalities in MIA offspring[229,320,321]. Oral treatment of MIA offspring with the human commensal *Bacteroides fragilis* not only restored peripheral immune homeostasis and corrected gut permeability but ameliorated several aberrant behaviors [320]. Finally, intraperitoneal injection of naive mice with a metabolite (4-ethylphenylsulfate) that was observed to be increased in MIA offspring and restored by *B. fragilis* caused certain behavioral abnormalities, suggesting that gut bacterial effects on the host metabolome impact behavior [320].

The mechanism by which an altered microbiome may influence brain development and cognition in offspring born to *H. bakeri* infected mothers is unknown. However, several putative mechanisms and pathways by which the microbiome influences CNS processes include the vagus nerve, HPA axis, the immune system, or by way of microbial metabolites such as short-chain fatty acids (SCFAs)[322,323]. The primary and most direct way for the microbiota to influence the brain is via the vagus nerve[322,324]. Of great interest to my study, stimulating the vagus nerve enhances memory[325-327], facilitates hippocampal neurogenesis, increases expression of BDNF[327-329], and enhances hippocampal LTP[327], all of which I observed in offspring of *H. bakeri* infected dams.

Specific bacterial strains have been shown to influence vagus nerve signaling, to communicate with the brain, and alter cognition. For example, greater abundance of SCFA producers, such as *Lactobacillales,* are associated with better vagus function[330]. Further, probiotic supplementation of *Lactobacillus* species was associated with increased vagus nerve activation[330,331], improved spatial memory and cognition[332] and decreased anxiety[332,333] in

rodents. Of great interest, it has been demonstrated that *H. bakeri* significantly raises *Lactobacillus* species abundance in the intestinal microbiome of infected mice and this correlated positively with a heightened Treg response[154,155]. Further, maternal *H. bakeri* infection significantly increased the abundance of *Lactobacillus* species in the infected mother as well as in their uninfected neonates[105] and three-month old offspring[9].

In addition, it was observed that the altered microbiome in response to maternal *H. bakeri* infection was associated with increased production of SCFAs, including acetate and butyrate, in the dam's milk[9], in the neonatal stomach[105] and in the feces and serum of three-month old offspring[9]. The SCFAs acetate, propionate, and butyrate are the main metabolites produced in the colon by bacterial fermentation of dietary fibers and resistant starch[334]. Growing evidence supports the idea that SCFAs regulate CNS processes through both direct and indirect mechanisms that can ultimately affect neurodevelopment as well as host cognition and response to stress[323,335,336]. SCFAs can cross the BBB via monocarboxylate transporters located on endothelial cells or they can directly activate the vagus nerve[337,338]. SCFAs suppress inflammatory responses in the intestine and other organs by inducing Treg differentiation and can also dampen neuroinflammation by affecting glial cell maturation morphology and function[322,334,339,340]. They also regulate the formation of the BBB, microglial maturation, and synaptic plasticity and modulate levels of neurotrophic factors, such as BDNF, increase neurogenesis, promote neuronal homeostasis and function, and have been found to improve cognition[322,323,334,341,342]. Further, SCFA administration reduced HPA axis hyperactivity by significantly attenuating the cortisol response[341,343,344], and reduced anxiety-like behaviour[345].

The altered microbiome in response to maternal *H. bakeri* infection may also influence offspring cognition via immune pathways. Specifically, as an increase in the abundance of *Lactobacillus* bacteria[154,346], as well as SCFAs[347], is associated with an expansion of Tregs and increase in TGF-β, it is possible this could in part explain the up-regulation of the TGF-β signalling pathway in the hippocampus of offspring born to *H. bakeri* dams, which I hypothesized was associated with the up-regulation of genes associated with neurogenesis, gliogenesis and myelination, and promoted a pro-cognitive phenotype of glial cells that was associated with BDNF production. Additionally, the HPA axis is influenced by the immune system[348] and the microbiome[349], and the HPA axis is known to influence hippocampal

function and memory[350,351]. Thus, this may be an additional pathway by which offspring spatial memory is influenced in this model.

Taken together, the ability of a maternal *H. bakeri* infection to alter the gut microbiome of their offspring, specifically by increasing the abundance of *Lactobacillus* species and production of SCFAs[9,105], provides an additional mechanism behind the enhanced cognition I observed, possibly via activation of the vagus nerve and/or SCFAs crossing the BBB, which may influence glial cell development and function and stimulate the production of BDNF to promote LTP and spatial memory. This altered microbiome may also influence the immune system of the offspring, as well as their HPA axis, providing multiple pathways by which the microbiome might influence CNS processes in this model. It would be of great interest to determine if the microbiome does have a significant role in mediating the brain and behaviour changes observed in offspring in response to maternal GI nematode infection. As such, it would be interesting to attempt microbiota transfer therapy, whereby the gut bacteria from offspring of infected dams are transferred to offspring of uninfected dams, to determine whether this enhances their spatial memory ability. Further, to assess if the HPA axis is influenced, one could start by assessing serum levels of corticosterone in the offspring, as the HPA axis regulates circulating levels of this glucocorticoid hormone[352].

6.2.3. Maternal care

I cannot rule out that *H. bakeri* infected mothers had alterations in their approach to maternal care, which has been previously shown to influence neurodevelopment and behaviour of offspring[353]. Measures of maternal care in rodents typically include nest attendance, anogenital and body licking and grooming of pups, nursing (sometimes further specified into blanket, low-arched back, high-arched back, and passive nursing), nest building, and retrieval of pups when they are displaced from the nest[354]. It has been shown that maternal exposure to a viral mimic resulted in reduced pup licking and grooming behavior which was associated with depression-like behavior in the offspring[355,356]. Further, previous studies found that offspring of mothers showing high levels of pup licking and grooming and arched-back nursing have increased expression of the NMDAR subunit, NR2A, and BDNF mRNA, as well as enhanced hippocampal LTP and spatial memory[357,358]. These findings are consistent with the observations

I made in offspring of *H. bakeri* infected mothers, indicating that infection-induced changes in maternal care might also explain the brain and behaviour changes I observed in this model. To the best of my knowledge, no studies in humans or lab models have assessed whether a maternal GI nematode infection may alter the way in which the infected mother interacts with and cares for her offspring. It would thus be of great interest to assess this in a cross-fostering experiment.

6.3. Broader perspectives of maternal GI nematode infection on offspring outcomes

I have provided evidence that maternal *H. bakeri* infection during pregnancy and lactation resulted in enhanced spatial memory in two and three week old juvenile male and female offspring, and this was retained in adult female, but not male, offspring. My data suggests that maternal *H. bakeri* infection promotes a Th2/Treg biased neuroimmune environment in the hippocampus of juvenile male and female offspring, which may promote spatial memory. Given that the male sex hormone, testosterone, dampens Th2 immune responses [359-362], I hypothesized this may dampen the Th2/Treg biased neuroimmunity in adult male offspring of *H. bakeri* infected dams, explaining why I no longer observed enhanced spatial memory. I would not have expected there to be a strong influence of sex hormones on the immune response in the two-three week old juvenile offspring, as circulating sex hormones are at low levels in these prepubescent mice[363]. It would be of great interest to explore the neuroimmune system in adult offspring of *H. bakeri* infected dams, and to determine whether sex hormones are the driving factor behind these observations.

My data indicates the influence of maternal infection on offspring spatial memory may be particularly strong during the juvenile stage, and this could be evolutionarily advantageous. Wild mice start venturing away from their nest at two-three weeks of age, to learn how to navigate their environment and find food[364,365]. During this period, spatial navigation and memory is highly relied on as it is necessary for foraging, mapping their environment and remembering how to find their way back to their nest[364]. It is also important for predatory avoidance, as juvenile mice are at increased risk of predation[366], and mice must be able to search for the safest shelter to avoid a predator[367]. As I found enhanced spatial memory in two and three-week old offspring in response to maternal GI nematode infection, it can be hypothesized this may increase the

fitness of offspring, as this is an age where they are highly vulnerable and heavily rely on spatial memory for survival. Further, I found enhanced spatial memory was retained in adult female offspring of GI nematode infected dams, but not male offspring. It may be evolutionarily advantageous for females to retain these enhanced fitness measures, as males are typically more dispensable in a population, and the reproductive effort and parental investment are more costly for female mice than for males[368]. Further, in wild mouse populations, female mice bring pups on foraging trips to teach them more about diet selection and food location, during which the environment must be navigated cautiously, to avoid predators[364,369]. Thus, it is possible that, in addition to enhanced spatial memory being beneficial to the survival of the individual, it may also aid in the survival of offspring.

It is worth highlighting the contrasting consequences of prenatal exposure to bacteria and viruses, or their mimics (LPS and poly IC, respectively), compared with GI nematodes. Maternal infection with bacteria or viral pathogens has been identified as a risk factor for inflammation-associated neurodevelopmental disorders like ASD and schizophrenia[43]. In MIA models, offspring present with deficits in social interaction[43,263,264], sensorimotor gating[225,237,267], cognition[55-57,59,60,256,268,370], and the occurrence of repetitive behaviours[263,264,266], all of which are relevant to ASD and schizophrenia[43]. Of particular relevance, exposing pregnant dams to LPS[45,48,54-57,223,256,268,370,371], polyIC[58,248], or *Escherichia coli* infection[59,60,241], increased levels of proinflammatory cytokines (IL-1β, IL-6, and TNFα) in the hippocampus, activated microglia and astrocyte phenotypes associated with neuroinflammation, reduced oligodendrocyte numbers and myelination, and impaired hippocampal neurogenesis, LTP and spatial memory in offspring. Of further interest, when the anti-inflammatory drug, N-acetylcysteine[56], the anti-inflammatory cytokine IL-10 [60,241], or IL-1 receptor antagonist (IL-1ra) [223] were administered to the dam, pro-inflammatory cytokines and microglia activation in the brain was decreased, and myelination, LTP and spatial memory deficits were rescued. In contrast to these findings from MIA models, my data demonstrates that maternal *H. bakeri* infection induced a Th2/Treg immunoregulatory environment in the developing hippocampus, which was associated with upregulated genetic markers of neurogenesis, oligodendrogenesis and myelination, and enhanced LTP and spatial memory. It would be interesting to consider, if, similar to administration of anti-inflammatory agents, a maternal *H. bakeri* infection would be able to prevent cognitive deficits in an MIA model by dampening harmful neuroinflammatory responses. This may be of particular

interest to researchers focused on prevention of inflammation-associated neurodevelopmental disorders like ASD and schizophrenia. Of note, however, before considering this avenue, it would first be important to assess other behaviours in offspring born to *H. bakeri* infected dams.

Of interest, enhanced anxiety[265] is associated with MIA offspring, and my data indicated that maternal *H. bakeri* infection may influence anxiety. I observed that 16-day old male and female offspring of *H. bakeri* infected mothers explored a novel environment less compared to offspring of uninfected mothers, which can be an indication of heightened fear or anxiety[372]. At three-weeks of age, I did not observe any differences in anxiety-like behaviour in offspring during the BMT, however, at three-months of age, female offspring of *H. bakeri* dams appeared less anxious compared to females of uninfected dams, as indicated by fewer fecal pellets when they were first introduced to the maze, and increased exploration during the training phase. Although these results are intriguing, the BMT was designed to assess spatial memory, not anxiety. As such, it would be helpful to utilize the elevated plus maze, which has been validated as an assessment of anxiety in preclinical studies[265], in order to provide more conclusive data on the influence of maternal *H. bakeri* infection on anxiety behaviour in offspring. Considering anxiety behaviour is largely reliant on the amygdala, and also involves the hippocampus, hypothalamus and thalamus[373], the indication that maternal *H. bakeri* infection may be influencing anxiety behaviour of offspring, further highlights the need to explore other brain regions and behaviours in this model.

Further, considering a number of behaviours in MIA offspring are influenced by the species (rat vs. mouse), strain, immunogen used, timing of immune insult, and pup age during testing[43], I acknowledge there are still many important factors to consider when trying to understand the impact a GI nematode infection may have on offspring behaviour. For instance, it would be important to know how the infection protocol used (e.g. single, vs. challenge vs trickle infection with *H. bakeri*), and timing of the infection during gestation, may influence offspring outcomes. It would also be important to consider other GI nematodes, such as *N. brasiliensis,* as protection against *N. brasiliensis* is also associated with type 2 immunity[374], and previous studies have shown maternal infection with this nematode alters offspring immunity and microbiome[101,102].

I have largely focused on the influence of maternal GI nematode infection on offspring behaviour. However, when considering whether prenatal exposure to GI nematodes is beneficial or harmful to the health of the offspring, I acknowledge the answer will not be straightforward as there is an overwhelming number of factors to consider, and it is likely that there will be both positive and negative impacts on the offspring. For example, I observed both juvenile and adult offspring had reduced mass and crown-rump length in response to maternal *H. bakeri* infection. Although growth stunting can be identified as a negative outcome, it has previously been shown that maternal *H. bakeri* infection protects adult offspring from high-fat-diet-induced obesity through altered microbiota and SCFAs[9], which would assumingly be a positive outcome. Further, I observed enhanced resistance to direct *H. bakeri* infection in offspring of *H. bakeri* dams compared to offspring of uninfected dams. As GI nematodes typically do not impose life-threatening risk to their hosts, unless present in large numbers[28], the ability to control infection, would assumingly be beneficial to the overall health of offspring. However, it would be important to understand the full extent of how a maternal GI nematode infection influences the development and function of the offspring's immune system. For instance, wild rodents[375] and humans[376,377] in GI nematode endemic settings are exposed to a plethora of pathogens, including other helminth species, bacteria, viruses and protozoan parasites, and it has been proposed that maternal GI nematode infection may compromise the ability of offspring to protect themselves against certain pathogens[35,378]. Although increased susceptibility to pathogens could negatively impact the health of the offspring, it has been proposed that the tolerogenic effects of prenatal exposure may reduce inflammation-induced pathology, resulting in an improved outcome for the offspring[35]. Additionally, it has been proposed that maternal GI nematode infection may be beneficial in the prevention of chronic inflammatory/ hyperimmune-associated disorders[80,106].

In countries where GI nematodes are ubiquitous in human populations, the incidences of "hyperimmune-associated disorders", including multiple sclerosis (MS), IBD, eczema, asthma, and allergies, have not seen the dramatic increase that modern societies have[79]. These rises in hyperimmune-associated disorders cannot be accounted for by changes in genetic susceptibility alone, and although many environmental factors have been implicated in these increases (e.g. industrial toxins and chemicals), the associations of single agents with hyperimmune-associated disorders have been relatively weak[106]. The hygiene hypothesis, also referred to as the biome

depletion theory, or the "old friends" theory, suggests that "biome depletion" – loss of commensal microbial and multicellular organisms, such as GI nematodes – has left our immune system profoundly over-reactive, with a strong propensity to react against a wide range of non-pathogenic self and non-self antigens, which contributes to this hyper-immune epidemic[79,80,379]. In addition to epidemiological evidence that supports this theory[80], animal models and pre-clinical trials have suggested a beneficial effect of GI nematode infections on a number of autoimmune, IBD and allergic diseases[81], and there is increasing interest to utilize GI nematode therapy in humans as a means to prevent and treat hyper-immune diseases[79,80,82-86]. Thus, the central hypothesis is that immune destabilization due to the loss of GI nematodes and commensal microorganisms (a.k.a. biome depletion), coupled with environmental triggers and/or genetic susceptibilities, has led to a population-wide increase in the overall incidence of disease associated with immune hypersensitivity[80].

Shaping of the immune system starts *in utero* and prenatal as well as early postnatal developmental stages seem to represent a certain "window of opportunity" for environmental influences, such as GI nematodes, to prevent hyperimmune-associated disorders[35,79,87,88,106]. Although this is a promising avenue, our understanding is poor, and a combined effort by clinical and experimental research is needed. Of interest, one study in rodents observed that maternal infection with the trematode *Schistosoma mansoni*, protected offspring from respiratory allergy, however, this protection only occurred when *S. mansoni*-infected mothers were mated during the Th1, or regulatory phase of infection[107]. Further, a human study found anthelmintic treatment of Ugandan mothers increased the prevalence of infantile eczema and respiratory wheeze compared with that seen in a placebo-controlled group[95,108]. Findings from my thesis may also be of interest, particularly with regards to MS. MS is a neurological disease characterized by an inappropriate autoimmune response against myelin, that ultimately leads to demyelination along with inflammation, axonal loss, and reactive glial cells, and a range of symptoms including paralysis, loss of vision and co-ordination[380]. All current MS therapies are either immunomodulatory or immunosuppressive, highlighting the central role of the immune system in MS pathogenesis[381]. MS prevalence has an inverse relationship with GI nematode prevalence, with Western Europe and North America having the highest prevalence of MS, while Asia, Africa, and the Middle East have the lowest prevalence[382]. Evidence from rodent models demonstrate a role for pre-existing GI nematode infection in preventing or limiting the onset of

MS-like symptoms and neuroinflammation[381]. Importantly, however, this has never been studied in the context of a maternal GI nematode infection. The data I have presented in my thesis suggests that a maternal *H. bakeri* infection is able to regulate the neuroimmune environment in the hippocampus of offspring. Additionally, my RNA-seq data indicates maternal *H. bakeri* infection promotes oligodendrogenesis and myelination in the developing brain, and may also promote remyelination, which is critically dependent on the genes *Myrf*[383], *Zfp488*[384], *Klf9*[385], *Stat3*[386] and *Olig1*[387,388], all of which were significantly up-regulated in response to maternal infection. There are well established MS models in rodents, with the experimental autoimmune encephalomyelitis (EAE) model being the most studied[389]. It would be of great interest to assess whether a maternal *H. bakeri* infection is able to prevent neuroinflammation and demyelination in offspring exposed to EAE. If prenatal exposure to a GI nematode infection prevents the onset of hyperimmune-associated disorders, this could lead to a paradigm shift that highlights not only the negative but also positive impacts of GI nematode infections on their host and on the next generation.

6.4. Conclusion

It seems counterintuitive to think that a GI nematode infection may have benefits to its host, but this possibility is not without precedent. GI nematodes have co-evolved with their hosts and have shaped many aspects of host physiology, metabolism, and immunology. They typically do not impose life-threatening risk to their hosts, unless present in large numbers. The biome depletion theory acknowledges that the potential benefit of GI nematodes is attributed to their ability to regulate their hosts immune system. Based on this, efforts are currently underway to utilize nematodes or their excretory/secretory products for the treatment of IBD, asthma, allergies, MS, obesity and diabetes [9,82-86,390]. It would be important to consider the perinatal period, as it is likely that exposure to GI nematodes during development may have the greatest influence on the life-long function of the immune system[35,379].

Further, it may also be important to acknowledge that GI nematodes may have unexpectedly broad impacts on many areas of human and animal biology. My study provides evidence for the first time that exposure to GI nematodes during pregnancy and lactation promotes hippocampal LTP and spatial memory, possibly through transfer of a Th2/Treg

immune phenotype from the infected dam that protects the offspring from direct infection and extends to their developing brain. Considering spatial memory is an essential aspect of survival for mice[391], enhancement of spatial memory might thus be evolutionarily advantageous. This may be particularly important during early life, where young pups must leave their nest and learn how to navigate the world and survive on their own. If similar results are proven to be true in humans, this could lead to a paradigm shift that highlights not only the negative but also positive impacts of GI nematode infections on their host and on the next generation.

It may be that maternal exposure to GI nematodes is an important contributor to our immune education, allowing protection against the onset of autoimmune disease, allergy, and infection, and that it may even benefit our brain development and cognitive function. However, despite my findings in this thesis, which highlight a benefit of maternal GI nematode infection for offspring spatial memory, there are still many factors to consider before one can determine whether prenatal exposure to GI nematodes is beneficial or harmful to the offspring. Further, it is important to remember that in human populations, many GI nematode infections occur in areas of high population density and poor sanitation which leads to high parasite burdens and host pathology. This is often combined with malnutrition and coinfections with other pathogens such as malaria, and as such, under these conditions, maternal GI nematode infection may be associated with negative influences on the infected mother and child health. Alternatively, it is possible that prenatal exposure to GI nematodes could be beneficial in wealthy societies that have access to medical care to help control worm burden and nutritional status of the mother, allowing benefits of GI nematodes to be optimized for preventing hyper-immune disorders and possibly enhancing cognitive function. As such, a combined effort by clinical and experimental research is necessary to understand the significance of GI nematode exposure for the overall health of the mother and child, while taking into account important factors, such as nutritional status, infection intensity and susceptibility to other pathogens.

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