Investigating the Role of Microglia in Gestational Intermittent Hypoxia-Induced Cognitive Deficits in Rats

By

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ABSTRACT

The maternal-fetal environment during pregnancy plays a tremendous role in shaping our lives. Sleep disordered breathing (SDB) is experienced by over 14% of the pregnant population and is comorbid with prenatal complications including hypertension. It is associated with increases in inflammatory cytokines similar to maternal immune activation models that induce cognitive and behavioral impairments in offspring. Despite its prevalence, the long-term consequences of maternal SDB on developing offspring are virtually unexplored. Microglia, the immune cells of the central nervous system, are responsible for proper brain development and homeostasis, clearing debris, pruning of dendritic spines during postnatal development, and responding to inflammatory challenges such as those induced by SDB. Whether maternal SDB alters offspring microglial function is unknown. We explore this by exposing rat dams to intermittent hypoxia during gestation (GIH), a model of SDB during pregnancy. In chapter 3, I show that while GIH does not alter microglial number or phagocytic capabilities during postnatal development, it does alter microglial morphology in a manner that may reduce their surveillance. Microglial surveillance can be blunted by norepinephrine acting on the ß-adrenergic receptor, and SDB has been linked to increased norepinephrine levels. In chapter 4, I utilize a ß-adrenergic receptor antagonist commonly prescribed for hypertensive pregnancies concurrent with GIH exposure to investigate their combined effects on offspring cognitive and social behaviors in adulthood. Collectively, these studies characterize the effects of GIH on postnatal microglial development and expand our understanding of how prenatal SDB may induce cognitive and behavioral deficits in the offspring.

CHAPTER I

COMMUNICATING RESEARCH TO NON-SCIENCE AUDIENCE FOR THE WISCONSIN INITIATIVE FOR SCIENCE LITERACY

Preface:

I would like to thank the Wisconsin Initiative for Science Literacy (WISL) for their efforts to promote literacy in science, mathematics, and technology. One of my core goals as a scientist is to effectively present my findings/results to a diverse audience with limited scientific background. We as scientists tend to live in a bubble where we only interact with those of similar education levels. However, we are but a small part of a much larger society which we depend on. For science to continue to be accepted and funded, the general public must understand what we do and, more importantly, WHY we do it. Therefore, I strive to articulate complex topics in an understandable manner and am grateful for the opportunity to do so within my doctoral thesis.

Sincerely,

Andrew Knutson

The Importance of Thorough Research

What do smoking, alcohol, sushi, and changing the litterbox all have in common? They're all things people are commonly told to avoid during pregnancy. Nowadays, we know that there are certain things pregnant people need to avoid. Similarly, we know that young children should not consume alcohol because it can affect their development, and that some drugs like painkillers and antianxiety medication affect women differently than men. However, up until the early 1960s, when a drug was found safe in adult men, it was usually prescribed to any adult without consideration for sex or other complications such as pregnancy. It took a terrible disaster known as the Thalidomide tragedy to change that view around the world.

Thalidomide was originally prescribed as a sedative or anti-nausea medication for treating common ailments like headaches, flu, or cold. Morning sickness is a feeling of nausea commonly experienced by pregnant people, and unsurprisingly, thalidomide was prescribed to tens of thousands of pregnant women to treat this symptom. Sadly, when prescribed during early pregnancy, thalidomide led to horrible birth defects; the most striking being limb deformities. It took around 5 years before thalidomide was identified as the culprit, and in response to the thalidomide tragedy, new testing regulations were put in place around the world mandating any new drug must be tested for both safety and efficacy in its target population before being prescribed to them. This means that if a drug is going to be prescribed to pregnant people around the world, it must first be proven safe in a group of pregnant people. However, when this law went into effect, there were already so many drugs being used in pregnancy that had never previously been tested, and not nearly enough money or time to go back and test them all. Any current drugs therefore

were “grandfathered in” and considered safe until proven otherwise. This law went into effect in 1962, but it still took years before commonly used drugs such as cigarettes and alcohol were proven dangerous during pregnancy. For example, it wasn’t until 1973 that alcohol was formally linked to fetal alcohol syndrome, and even then, it was only in regard to alcoholic pregnancies. It took another 5 years until low amounts of alcohol were linked to negative effects in the child as well.

Alcohol, smoking, sushi, and changing the litterbox were all once considered harmless for pregnant people, and it took someone doing further research to identify the issues they cause. If I were to suggest adding sleep apnea to that list, the first thing you would probably think of is an older man falling asleep in his chair after a meal and snoring away, but surprisingly, around 14% of otherwise normal pregnancies and up to 60% of obese pregnancies also experience some form of sleep disordered breathing (SDB) by the third trimester. Despite this, sleep apnea, a form of sleep disordered breathing, is rarely touched upon in the doctor’s office during visits with pregnant women. Yes, they may ask questions like “Are you getting enough sleep?” or “Are you having issues sleeping?” but rarely do these conversations progress beyond advice on sleeping positions to minimize discomfort. This is because the consequences of SDB during pregnancy are not well known or characterized despite impacting such a huge number of births. Someone still needs to do some thorough research to figure out if there are any consequences, and that someone is my lab.

When I first learned that I could do my PhD in a lab that studies SDB during pregnancy, I immediately knew it was the lab for me. I entered graduate school hoping to study some sort of exposure during pregnancy, and how it would impact the baby throughout life.

Additionally, my father had just been officially diagnosed with sleep apnea in the months before graduate school and my mother had been raving about how much easier it is to sleep in the same room as him now that he was on the therapy for it. After learning more about the prevalence of SDB during pregnancy and how little we know about the long-term consequences, I knew it was the lab for me!

Researching Sleep Disordered Breathing During Pregnancy

SDB during pregnancy is commonly associated with negative short-term birth outcomes including low birth weight and preterm labor, however the long-term consequences on the offspring are virtually unexplored. To study these long-term consequences without having to wait years for a human baby to grow up, we study the effects of SDB during pregnancy using rats. Rats are commonly used to test different drugs or exposures in place of humans because of their small size for housing purposes and because they are relatively inexpensive. Their bodies function very similarly to humans, and therefore, a lot of research outcomes in rats can easily translate to humans. When an individual has SDB, they stop breathing for short periods of time each hour, reducing their oxygen intake, and intermittently lowering the amount of oxygen in their blood. This happens many hundreds of times each night, during sleep. Because rats don’t naturally have SDB, we mimic it by housing them in special chambers where we can manipulate the amount of oxygen in the air they breathe. We expose pregnant rats to a mild to moderate level of SDB (15 episodes of reduced oxygen per hour) for 8 hours a day during their sleep period during the second half of their pregnancy to mirror a realistic case in humans. For a fair comparison, we test pairs of pregnant rats, one of which undergoes the SDB treatment while the other is kept in an identical chamber with identical airflow and noise levels, but

without changes in oxygen levels. After they give birth, we raise the offspring in identical conditions. When the SDB offspring grow into adult rats, we test them in a variety of aspects, but the particularly important aspects for my research are cognitive and behavioral endpoints. They are tested for how well they can think and remember, and whether they socially interact “normally” as compared to other rats whose mothers did not have SDB during pregnancy.

For the sake of time, I’m only going to go into detail on one cognitive endpoint: spatial working memory. Spatial working memory is a measure of how well someone can recognize where they currently are, and where they have already been. Think of it like being able to tell directions, but since we are testing a rat instead of a human, it is on a very basic scale. To test spatial working memory, we use a three-armed “maze” in the shape of a Y called the “Y-maze” test (Figure 1). This test uses the rat’s natural urges to explore new places to test whether it can remember where it is and where it has already been. The rat is placed in the end of one arm, designated arm A. The naturally inquisitive rat will leave the first arm and enter a second designated arm, B. At this point, it has been in all arms except arm C. If it has proper working spatial memory, it will leave arm B and enter arm C, the only arm of the 3 that it has yet to explore. However, if it has impaired spatial memory, it has a 50-50 chance of leaving arm B and entering arms A or C. By performing the test for 10 minutes and counting the number of 3 arm successes (ABC) compared to the total number of trials (ABC+ABA), we get an idea of how strong their spatial working memory is.

The Consequences of SDB During Pregnancy

Now remember, these offspring are identical to their comparison litter with one exception. Their **mother** had SDB during her pregnancy. When the offspring become adults, we find that the adult **male** offspring of SDB pregnancies have impaired spatial working memory, as they lack the ability to choose the third arm above the 50-50 chance level. We also see that they have impaired long-term memory and lack an interest in socially interacting with rats they have never previously met. Each of these different cognitive and behavioral endpoints are controlled by different regions of the brain.

One such region towards the front of the brain is called the prefrontal cortex. In this region, there are lots of neurons, the cells of the brain that store and process information. They store information in the form of connections between neurons called synapses. These connections constantly change based on experience- they can become stronger, weaker, develop a new synapse, or disappear entirely. My lab has taken brains of these SDB rat offspring and found an increase in the number of these synapses in the prefrontal cortex compared to non-SDB rat offspring.

Changes in the number of synapses are involved in many developmental and neurological disorders including autism, schizophrenia, Alzheimer's disease, and in cases of prenatal infections. During normal rodent brain development, the number of synapses generally increases over the first few weeks of life when virtually everything is a new experience. Then, the excess, unnecessary synapses are typically pruned away around the onset of puberty, after which time, synapse numbers reach normal adult levels that are generally maintained throughout life. The number of synapses and their pruning is a carefully controlled developmental process that involves a cell type called microglia that contribute to synapse maintenance.

Microglia

Microglia are immune cells that reside in your brain. They enter the brain through the blood during the first trimester of fetal brain development and continue colonizing it until the blood-brain barrier develops and prevents additional cells from entering the fetal brain. The blood-brain barrier is a protective blockade formed by a group of tight cells along the outside edge of blood vessels in the brain that serve to prevent chemicals and cells from passing into the brain (Figure 2). After this barrier forms, microglia become a self-renewing population of cells, which means that new microglia are formed throughout life, from the existing population that entered the brain during embryonic development. Because of this, any early-life insults to microglia can lead to life-long changes in their function.

Microglia are extremely versatile little cells that play several roles in the brain throughout early development. They are responsible for clearing out dead cells and other debris from the brain's environment and for keeping it tidy. They provide various chemicals required for a healthy brain environment and they are responsible for identifying and removing extra synapses between neurons, through a process called pruning. These are the same synapses that we see more of in SDB rat offspring.

Because of the increased number of synapses in adult male SDB offspring, I suspected that something might have happened to microglia in early development to impair their pruning ability. When thinking about ways in which we could get **less** pruning of synapses by microglia, I came up with three possible hypotheses: 1) SDB rat offspring have less microglia, therefore there are less around to prune synapses, 2) SDB rat offspring microglia have impaired ability to phagocytose (engulf and remove) the synapses that

need to be pruned, or 3) SDB rat offspring microglia are unable to properly survey their environment to find the synapses that need to be removed.

Counting Microglial Number

Counting every cell in the brain would be a ridiculous endeavor. We're talking billions of cells. So instead, I utilized a technology called flow cytometry to quickly identify a fraction of the cells, and then extrapolate from there. I took the brains from several SDB and non-SDB rat offspring at 3-, 14-, 21-, and 28-days post birth and labeled them using a microglia-specific fluorescent marker. Then the flow cytometer pulls the cells up through a narrow tube one at a time and tests their fluorescence using lasers to count the number of cells that shine with that specific marker (Figure 3). We can then determine the number of microglia relative to all brain cells at each age, to see if microglial numbers differ from normal in SDB rat offspring.

I ended up finding no difference in microglial number at any timepoint I looked at, in SDB offspring (in either sex). However, I did detect the expected developmental increase in microglial cell number in early postnatal brain development in rats that had been previously shown in mice. Rodent microglia undergo a rapid increase in number over the first two weeks of postnatal life. This is followed by a gradual decrease during the third and fourth postnatal weeks where microglia cell numbers reach healthy adult brain levels, often between 5 and 15% of total brain cells. From these results, I concluded that my first hypothesis was false- SDB rat offspring do not have fewer total microglia than control offspring.

Examining Microglial Phagocytosis

To test whether SDB offspring microglia have a generalized impairment in phagocytosis, or “cellular engulfment”, I did a few different experiments. First, I isolated microglia from 3-day old SDB and non-SDB rat offspring brains and grew them in cell culture to allow them to replicate over time to reach a sufficient number for experimentation. I then exposed the cultures to fluorescent yellow-green latex beads that can be engulfed by a microglial cell, the numbers of which can be quantified using a flow cytometer. Microglia that have engulfed a particle will fluoresce yellow-green. When I compared the SDB and non-SDB offspring microglia, I found no evidence of reduced phagocytosis in SDB offspring microglia.

The exact mechanisms underlying how microglia phagocytose or engulf and remove neuronal synapses are still being identified. Each synapse, or connection between neurons, has a pre-synaptic and post-synaptic side. Think of a synapse like the cord connecting your DVD player to your tv. The pre-synaptic side is the DVD player which sends a signal across the synapse to the postsynaptic side, your tv. If you eliminate either side of the synapse, you lose the ability to transfer information. One molecule identified to be involved in post-synaptic neuronal phagocytosis is called complement. In the immune system, complement is used by macrophages to identify invading bacteria and other pathogens and target them for phagocytosis. In the brain, however, complement protein marks synapses that need to be eliminated, and microglia remove said synapses after binding to receptors on microglia that recognize and bind to neuronal complement. It is honestly quite amazing to think about how this system normally used to defend your body from invading pathogens is totally repurposed in the brain to help decide which memories to keep and lose based on how frequently that synapse or memory is used.

To determine whether complement-labeled material is more poorly phagocytosed by microglia from SDB offspring, I used the same yellow-green fluorescent latex bead, but this time, I coated the beads with complement. Additionally, rather than culturing microglia in a dish, where their development and response may differ compared to when they are in the brain, I used freshly isolated microglia from 28-day old rats, which is at a developmental time point close to when pruning of synapses is at its peak (close to puberty). Using flow cytometry to count the number of yellow-green microglia once again, I found no evidence for a reduced capacity to phagocytose complement-coated beads in SDB offspring microglia. While it is possible that I am just missing the mechanism that is impaired in SDB offspring (i.e., it is not complement-dependent), my results support the conclusion that microglia from SDB offspring do not have impairments in phagocytosis and indicate that my second hypothesis is also false.

Quantifying Microglial Surveillance

Just a couple decades ago, microglia were believed to be unremarkable cells that existed in the brain in a static state, to protect against invading pathogens. In the early 2000's, however, a landmark paper appeared where they thinned the skulls of mice and observed microglia moving in real-time under undisturbed conditions. It turns out that microglia are incredibly dynamic cells; they constantly survey their environment by extending and retracting their processes to repeatedly contact synapses and monitor synaptic activity. In fact, it is estimated that microglia survey every synapse in the entire brain roughly every 2-3 hours; when a synapse is inactive for an extended period of time, microglia remove it.

However, microglial surveillance, it turns out, isn't a constant thing. It ebbs and flows throughout the day alongside your circadian rhythm. The hormone norepinephrine helps to control your sleep-wake cycle, rising immediately prior to waking up, staying high during the day, and dropping to low levels as you fall asleep. This same hormone blunts microglial surveillance causing most microglial activity to occur during periods of sleep – including synapse pruning. Microglia are typically described as having a "ramified" shape in the healthy unperturbed brain, where they have many long thin "fingerlike" processes that become shorter and stubbier in the "amoeboid" or rounded shape they acquire when the brain experiences an insult, like pathogen exposure, stress, injury or disease (Figure 4).

To determine whether microglial surveillance is reduced in our SDB rat offspring, I took brains of SDB and non-SDB rat offspring during their normal sleep periods. I made thin tissue slices of the prefrontal cortex, the brain region where we have identified increased neuronal synapses, and I fluorescently labeled the microglia using a marker known as Iba1, that is only present in microglia in the brain. Iba1 is a useful protein because it colors the entire microglial cell body including all of their long finger-like projections or processes. I used a high-resolution microscope to observe these incredibly tiny cells, whose average diameter is about 40µm, or 4 one-thousandths of a centimeter in size. I then used computer imaging software to outline the cell body and all the processes of the microglia to calculate different morphological aspects including how often their processes branch off into new processes, how complex the cell is, etc. When compared to their non-SDB counterparts, our SDB rat offspring microglia display less branching, less complexity, and are less ramified suggesting a lower total surveillance. These data

support my third hypothesis that SDB rat offspring microglia may have an impaired ability to survey their environment and suggest that microglia from the SDB offspring brain may not be able to find or contact synapses that need to be removed as effectively as their non-SDB counterparts.

Conclusions

The thing no one really tells you about science is how often the results you predict just don't work out like you thought. When I first started out in my PhD. program, I had come from an industry lab where we tested medical devices for potential carcinogenic properties. In that type of job, negative data are the best data. Negative data mean that the medical device some company has put tens to hundreds of thousands of dollars into is **not** potentially going to cause cancer in someone. However, novel research for a PhD is quite different. You spend hours to days to weeks thinking up a question no one has ever asked before and planning the perfect experiment. You then spend weeks to months to even years testing the question, and a lot of the time the results end up negative. The reality is that science often doesn't cater to what is simple or easy. Biology is usually much more complex than we give it credit. When first starting out with the previously addressed hypotheses, I hadn't even thought of the third hypothesis. My potentials were "there are either less microglia or the microglia can't phagocytose as well". It took about three years of negative results and reading lots of papers from other labs before the third hypothesis that "maybe the microglia just can't sense or contact the synapses" came about. Overall, I set out to answer a question, and I did it. But science doesn't just stop there. Now we publish our results and someone – maybe us, maybe another lab – reads those results and it leads to their next question. As for me, I'll be taking what I've learned

about testing different exposures during pregnancy to an industry toxicology job where I'll be responsible for testing new medical devices and therapies for safety in animal models before they make it to humans. In the meantime, my lab will continue to work on getting sleep apnea added to that list of things doctors consider diagnosing and treating in their pregnant patients, because based on our rat studies, SDB is likely a risk factor for reduced cognitive function in male babies of mothers in whom SDB goes undiagnosed and untreated.

FIGURES

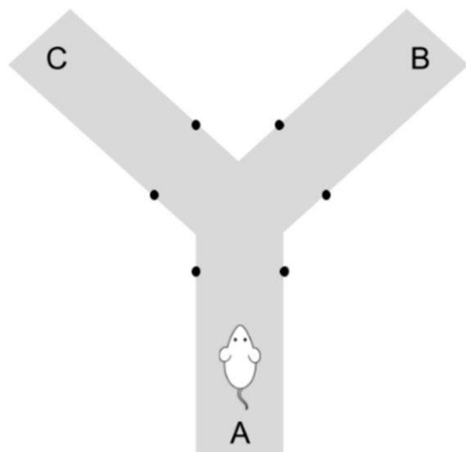

Figure 1: Y-maze Test depiction with rat starting in arm A.

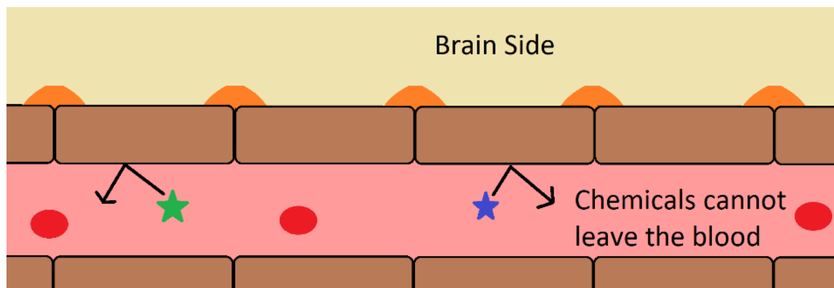

Figure 2 - Depiction of the blood-brain-barrier preventing chemicals from exiting blood and entering the brain

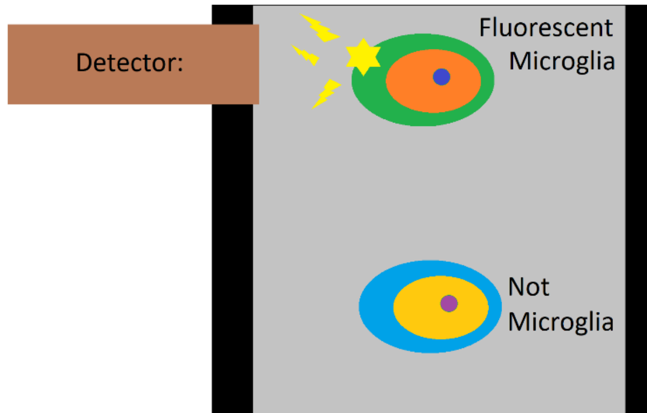

Figure 3: Depiction of a microglia and non-microglia cell going through the tube of a flow cytometer.

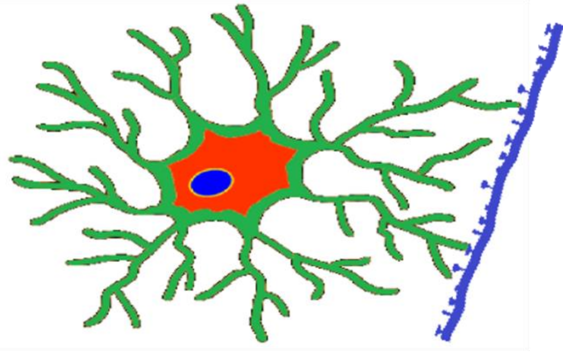

Figure 4: Depiction of a Ramified Microglia interacting with Synapses

CHAPTER II

INTRODUCTION

Portions of this chapter have been published in “All roads lead to inflammation: Is maternal immune activation a common culprit behind environmental factors impacting offspring neural control of breathing?”

Andrew O. Knutson, Jyoti J. Watters

Sleep Disordered Breathing

Sleep disordered breathing (SDB) is characterized by cessations in breathing during sleep. It can range from mild (5-15 apneas per hour; AHI) to more severe phenotypes of 30+ AHI (Senaratna et al., 2017), and affects approximately 17% of middle-aged men and 9% of middle-aged women (Peppard et al., 2013; Senaratna et al., 2017). The three main hallmarks of SDB are intermittent hypoxia (IH) where inhalation stops and oxygen levels decrease, hypercapnia where exhalation stops and CO_2 levels increase, and sleep fragmentation where upon reaching critical levels of hypoxia and hypercapnia, the body briefly wakes itself to restart breathing. Of these three hallmarks, intermittent hypoxia is known to recapitulate many aspects of SDB (Dewan et al., 2015).

During hypoxia, vulnerable cell populations enter apoptosis leading to systemic inflammation. SDB induces inflammation in adults by increasing inflammatory cytokines including IL-1β, IL-6, TNFa, CRP, while decreasing anti-inflammatory cytokines like IFNγ (Gaines et al., 2015; Hirotsu et al., 2017; Kong et al., 2018; Said et al., 2017). Alongside increased inflammation, SDB enhances sympathetic nervous system activity in adults (Nieto et al., 2009). Levels of the sympathetic hormone norepinephrine excreted in urine correlate with the severity of SDB, and SDB patients display an average norepinephrine increase of 60% (Ferrier et al., 2005). Importantly, IH can increase norepinephrine levels alone, increasing urinary norepinephrine levels by 50% in rats when tested after 4 weeks of exposure (Takahashi et al., 2018).

Sleep Disordered Breathing During Pregnancy

Despite only 9% of middle-aged women suffering from SDB, an astounding 14% of otherwise healthy pregnant women and up to 40% of obese pregnant women experience

SDB by the third trimester (Johns et al., 2020). Just like in nonpregnant populations, SDB is associated with increases in both inflammation and sympathetic nervous system activation. Pregnant women with mild cases of SDB display a significant increase in TNFα, IL-8, IL-1ß, and IL-10 cytokine levels alongside a trending increase in IL-6 levels (P=0.067)(Alonso-Fernández et al., 2021). Furthermore, their AHI during REM sleep correlates with the level of pro-inflammatory cytokines observed (Alonso-Fernández et al., 2021). Increased maternal serum cytokines is an essential aspect of maternal immune activation (MIA), and an increase in maternal serum IL-6 or IL-17a levels alone are sufficient to alter offspring brain development and behavior (Choi et al., 2016; Smith et al., 2007). Given the similarity in cytokines that are upregulated in both OSA and in various models of MIA, we propose that maternal sleep apnea may be another previously unconsidered, yet highly prevalent, model of MIA.

While less commonly studied than maternal inflammation, SDB also increases sympathetic nervous system activity in pregnancy. Pregnant women with gestational diabetes mellitis and SDB display increased normetanephrine, a metabolite of norepinephrine, in serum (Serednytskyy et al., 2022). Importantly, the sympathetic hormone norepinephrine plays a key role in initiating the stress response through the HPA axis suggesting an increase in prenatal stress levels among pregnant women with SDB (Sorrells et al., 2009). Just like maternal immune activation, prenatal stress leads to many long-term cognitive and behavioral impairments in the offspring (Michelsen et al., 2007; Weinstock, 2017).

The Consequences of Sleep Disordered Breathing During Pregnancy

With its links to maternal immune activation and prenatal stress, it's no surprise that SDB during pregnancy is associated with negative short-term birth outcomes such as preterm labor and low birth weight for gestational age; both of which are correlated with cognitive and behavioral deficits later in life (Carnelio et al., 2017; Ding et al., 2014; García Ventura et al., 2022; Kim et al., 2022; Silva et al., 2022). However, despite these negative birth outcomes and the consequences of prenatal stress and inflammation on offspring neurodevelopment, the long-term consequences of SDB during pregnancy are virtually unexplored. The studies presented in this thesis seek to address this gap in knowledge and broaden our understanding of not only what these long-term consequences are, but also the mechanisms by which they occur.

To determine the long-term consequences of maternal SDB during pregnancy without waiting decades for human studies, we utilize a rat model of maternal intermittent hypoxia exposure. We expose pregnant rats to intermittent hypoxia during the second half of gestation (GIH) mimicking a mild to moderate case of SDB in humans (15 induced apneas per hour). For comparison, we use rats exposed to gestational normoxia (0 induced apneas per hour) such that they undergo identical conditions as far as noise, light, airflow, and other stressors that may impact offspring development. After growing the offspring to adulthood, they are tested for a variety of endpoints.

While rats do not normally exhibit sleep apnea, our GIH male offspring display a significant increase in the number of spontaneous apneas (Mickelson et al. *in revision*) during sleep suggesting GIH offspring may experience sleep apnea and all the negative consequences that come alongside it like increased sympathetic activation and peripheral inflammation. Also, in adult male GIH offspring, we see deficits in cognitive and behavioral

areas including spatial working memory, long-term memory, and social approach behaviors. When further examining brain regions responsible for these behavioral deficits, we discovered a significant increase in synapse density of the medial prefrontal cortex of adult male GIH offspring (Vanderplow et al., 2022).

An Introduction to Synapses

Synapses are the connections between neurons typically consisting of a pre-synaptic zone on a neuronal axon and a post-synaptic zone on a neuronal dendrite commonly referred to as a “dendritic spine”. Synaptic strength is believed to correlate with how well developed or mature the dendritic spine appears morphologically. Smaller, thin spines are part of weaker, transient synapses while larger, mushroom-shaped spines are both stronger and more stable (Forrest et al., 2018; Runge et al., 2020). Synapses can be visualized and labeled in a variety of manners, but specific proteins such as synaptophysin and post-synaptic density 95 (PSD95) are typically used for labeling pre- and post-synaptic components, respectively (Comer et al., 2020; Wang et al., 2022).

Early in postnatal development, the brain forms synapses rapidly, reaching much greater numbers than those observed in adults (Forrest et al., 2018; Nimchinsky et al., 2002; Runge et al., 2020). During later postnatal development, excess, faulty, and underutilized synapses are pruned to reach normal adult levels (Forrest et al., 2018; Huttenlocher, 1979; Runge et al., 2020). This developmental pattern is critical for normal cognitive and behavioral development, as deviations in spine density are found in numerous neurological disorders including Autism Spectrum Disorder, Schizophrenia, and Alzheimer’s Disease (Forrest et al., 2018; Györffy et al., 2020; Hutsler and Zhang, 2010; Sellgren et al., 2019; Tang et al., 2014) as well as in both maternal immune activation

and prenatal stress models (Michelsen et al., 2007; Murmu et al., 2006; Radley et al., 2006; Xiao et al., 2021). Spine density is carefully controlled through both formation and pruning of synapses, and one cell type responsible for dendritic spine pruning is microglia.

An Introduction to Microglia

Microglia are the immune cells of the central nervous system that originate from the embryonic yolk sac (Utz et al., 2020). They colonize the developing rodent brain beginning around embryonic day 8.5 and continue until around embryonic day 13.5 when the blood-brain barrier forms (Ginhoux et al., 2013). At this point, they are an isolated, self-renewing population meaning any early-life insults, such as those experienced prenatally, result in life-long alterations to microglial function which may not become apparent until much later in life (Huang et al., 2018; Masuda et al., 2020). Additionally, microglia are incredibly long-lived as far as individual cells are concerned. In humans, carbon dating studies estimate that individual microglial cells last for years with the brain undergoing complete microglial turnover about once every 4 years (Réu et al., 2017). This further suggests prenatal insults will have long-lasting effects as the same microglia that experienced a prenatal exposure may still be around several years later. As GIH is one such early life insult, we seek to examine its effect on microglia across development. In early postnatal life, microglia rapidly proliferate over the first two postnatal weeks in rodents, and then proceed to decrease in number to reach adult levels around the fourth postnatal week (Nikodemova et al., 2015). In Chapter 3, we will determine whether GIH influences this developmental timeline by measuring relative microglial abundance between treatments throughout the first postnatal weeks.

Microglia play a variety of key roles in maintaining brain homeostasis. They phagocytose and remove cell debris while participating in the glymphatic system (Liu et al., 2022), provide key factors for neuronal survival and function like BDNF (Parkhurst et al., 2013), and respond to challenges such as inflammation (Rahimian et al., 2019; Riester et al., 2019). There are a variety of microglia-specific receptors in the CNS that allow them to play these roles. For example, microglia express CX3CR1, the fractalkine receptor, to monitor neuronal homeostasis (González-Prieto et al., 2021; Inoue et al., 2021). Activation of CX3CR1 by neuronally produced fractalkine dampens microglial activation and inflammation and promotes a healthy CNS environment. They also express P2Y12, allowing them to detect ATP gradients released by damaged and dying cells and migrate to sites of injury (Davalos et al., 2005; Mildner et al., 2017). Additionally, microglia utilize CSF1 receptor signaling for both survival and proliferation. By inhibiting CSF1R, microglia can be depleted over a period of days by up to 99% and remain depleted for weeks to months allowing for studies examining the role of microglia in various disease contexts (Coleman et al., 2020; Huang et al., 2018; Neal et al., 2020). Finally, microglia play a vital role in synaptic pruning of neuronal synapses throughout development and maintaining these synapses in adult life (Bohlen et al., 2019; Choudhury et al., 2020; Ji et al., 2013; Li et al., 2020). Because of their critical role in synaptic pruning coupled with the increase in dendritic spines observed in GIH offspring, we hypothesize that postnatal microglia are in some way altered by GIH exposure leading to the observed pruning deficits.

The Role of Microglia in Synaptic Pruning

How exactly microglia prune dendritic spines is still being elucidated. Early studies discovered a role for complement proteins including C1q, C3, and C4 where they bind

inactive spines, targeting them for elimination in an activity-dependent manner (Paolicelli et al., 2011; Parker et al., 2022; Schafer et al., 2012; Stevens et al., 2007; Wake et al., 2009). Further studies have implicated C4 overexpression in schizophrenia-induced over-pruning (Comer et al., 2020; Sellgren et al., 2019). Similar to complement proteins, phosphatidylserine residues have been observed bound to synapses before elimination and suggested to play a role in pruning. However, their elimination has been directly linked to C1q deposition as C1q knockdown increases phosphatidylserine deposition and decreases its engulfment by microglia(Parker et al., 2022). Regardless of the precise signaling molecules involved, microglia play a key role in phagocytosing dendritic spines, and complement plays a role in directing this removal. In chapter 3, we will test whether GIH male offspring microglia are impaired in their phagocytic abilities, which could lead to reduced pruning of dendritic spines.

In addition to complement, fractalkine was also identified by early studies as directly influencing synaptic pruning. In CX3CR1-knockout mice, there is an increase of PSD95 compared to wildtype controls and an increase in synaptic density due to a reduction in microglial pruning within the CA1 region of the hippocampus (Paolicelli et al., 2011). However, the role of fractalkine may be region specific, as while microglia have also been shown necessary for pruning of Purkinje cell synapses within the cerebellum, CX3CR1-knockouts have no impact on their pruning (Kaiser et al., 2020).

Microglial Pruning of Synapses without Phagocytosis

A more recent paper questions the idea that microglia phagocytose post-synapses and suggests alternative, phagocytosis-independent mechanisms of synapse removal. They found microglia contact roughly 3% of hippocampal dendritic spines, but only 15% of

those contacts also display the phagocytic marker CD68 (Weinhard et al., 2018). In these contacts, they found no evidence for phagocytosis of post-synaptic proteins and instead found small inclusions of pre-synaptic material which suggests partial phagocytosis, or trogocytosis, of pre-synaptic structures leaving the majority of the pre-synapse intact, rather than complete phagocytosis of post-synapses. Furthermore, knockout of the complement 3 receptor so frequently identified as key in post-synaptic phagocytosis has no effect on pre-synaptic trogocytosis (Weinhard et al., 2018). Lastly, when observed *in vitro*, approximately 10% of synapses contacted by microglia resulted in the synapse disappearing by the end of the imaging session. Importantly, the spine disappeared AFTER microglial contact had ceased, suggesting a microglia-initiated, but phagocytosis-independent pruning process. The mechanisms behind this process, however, are yet to be determined (Weinhard et al., 2018). Regardless of the precise mechanism of synaptic pruning, microglia have been shown to remove unnecessary synapses and their depletion leads to an increase in synaptic density.

Microglia Help Strengthen Synapses and Improve their Longevity

While microglia play a critical role in pruning of inefficient and unnecessary synapses, they also assist in synapse formation and strengthening existing synapses. Microglia produce neurotrophic factors including BDNF which is required for learning-induced synapse formation. Conditionally deleting BDNF from microglia in mice impairs long-term memory and fear responses (Parkhurst et al., 2013). These knockout mice result in a 50% reduction in synapse formation after two days of behavior training compared to wild type controls. Alongside neurotrophic factors, microglia frequently directly contact existing

spines and change their spine head morphology. Spine heads altered in this manner last significantly longer than those not contacted by microglia (Weinhard et al., 2018).

During early postnatal development, microglia also promote spine formation through direct contact with dendrites. In the developing cortex, neuronal dendrites generate filopodia, or immature spines, after a brief contact by microglia. This contact initiates a transient calcium elevation and accumulation of actin within the dendrite, resulting in spine head formation. These microglia-induced filopodia last longer than filopodia that form independent of microglial contact (Miyamoto et al., 2016; Weinhard et al., 2018). Overall, microglia play a key role in both synaptic pruning as well as strengthening of existing synapses throughout life. Therefore, the ability to properly survey their environment and contact surrounding dendrites is of paramount importance.

The Importance of Microglial Morphology

Assessing microglial surveillance begins with the topic of microglial morphology. For many years, microglia, as the macrophages of the central nervous system, have been described in binary states: quiescent or activated (Crain et al., 2013; Liu et al., 2019). Under homeostatic conditions, microglia typically display a very ramified morphology with lots of complex branching and many processes, but under inflammatory conditions such as disease, they exhibit a much more amoeboid morphology with fewer, shorter processes and a larger soma (Baldy et al., 2018; Tremblay et al., 2010; Walker et al., 2020). Because of this, morphology is often used as a pseudo-identifier of microglial activation state (He et al., 2021; Walker et al., 2020). Nowadays, we know that microglia can exist in a wide range of activation states and morphologies, not all of which are due to disease (Ohgomori et al., 2021; Walker et al., 2020).

Early studies utilized thinned-skull microscopy to observe microglia in the undisturbed CNS environment. They were the first such studies to show microglia are a dynamic population and not just sitting around and waiting for a pathogen or disease state to occur. Microglia are constantly surveying their environment, removing debris, and interacting with synapses such that they explore the entire brain parenchyma roughly every 2-3 hours (Nimmerjahn et al., 2005). Importantly, these studies were all done under anesthetized conditions, and it took nearly two decades before anyone thought to ask whether anesthesia has an impact on microglial morphology.

How Anesthesia Affects Microglial Morphology

By comparing thinned-skull microscopy in the awake animal to the anesthetized one, it was discovered that anesthesia results in a greater level of microglial surveillance due to hypoactivity of surrounding neurons, and this phenomenon is driven by local norepinephrine levels (Liu et al., 2019; Stowell et al., 2019). By stimulating norepinephrine release alongside anesthesia, they showed norepinephrine is sufficient to block anesthesia-related increases to microglial surveillance. Conversely, by decreasing norepinephrine production either through a sedative (dexmedetomidine) or by ablating norepinephrine-producing neurons in the awake animal, they could recapitulate the enhanced surveillance observed with anesthesia (Liu et al., 2019; Stowell et al., 2019). Microglia possess several adrenergic receptors capable of detecting norepinephrine including α1A, α2A, ß1, and ß2 (Mori 2002, Tanaka 2002, Farber 2005). By using pharmacologic inhibitors for each of these receptors, the ß2-adrenergic receptor was identified as regulating microglial surveillance in response to norepinephrine and then

reconfirmed through use of microglia-specific genetic knockouts (Liu et al., 2019; Stowell et al., 2019).

Microglial norepinephrine-ß2 adrenergic receptor regulation of microglial surveillance likely acts through modulating cAMP levels. ß2 adrenergic receptors are G_s-type receptors which increase cAMP levels upon activation by norepinephrine (Motiejunaite et al., 2021). Increased cAMP promotes protein kinase A (PKA) activity which inhibits phosphoinositide 3 kinase (PI3K) activity. PI3K inhibitors have been shown to decrease microglial motility in culture, and PI3K naturally increases Akt and mTOR activity which are linked to increased microglial motility and surveillance, hence why PI3K's inhibition results in an arrested surveillance phenotype (Cianciulli et al., 2020; Liu et al., 2017; Schneble et al., 2017). As further evidence for this pathway, norepinephrine can attenuate ATP-P2Y12 mediated microglial migration in culture, which is a G_i-type receptor that decreases cAMP levels leading to increased PI3K-Akt activity and hence a contradictory pathway (Cserép et al., 2020; Irino et al., 2008).

How Norepinephrine Impacts Dendritic Pruning

Norepinephrine is a sympathetic nervous system hormone that is circadian in nature, peaking just prior to waking and reaching a nadir during REM sleep (Choudhury et al., 2020). It is produced primarily in the CNS by locus coeruleus neurons which doesn't fully mature until around the third postnatal week (Nakamura et al., 1987). This leads to cyclical changes in microglial surveillance state over a 24-hour period, with increased surveillance during sleep when norepinephrine is at its lowest (Liu et al., 2019; Stowell et al., 2019). These periods of low norepinephrine free the microglia from their restraints, allowing greater surveillance and interaction with surrounding neurons. In fact, the majority of

dendritic spine pruning occurs during sleep when microglia are more surveillant (Choudhury et al., 2020; Yasumoto et al., 2021). Recall that complement 3 binds and targets excess synapses for elimination (Paolicelli et al., 2011; Parker et al., 2022; Schafer et al., 2012; Stevens et al., 2007; Wake et al., 2009). Complement protein levels peak immediately prior to the sleep period, and an increase in microglia-spine interactions can be observed during sleep (Choudhury et al., 2020; Yasumoto et al., 2021).

Recall that SDB increases circulating norepinephrine levels in the periphery (Ferrier et al., 2005; Green et al., 2021). Importantly, norepinephrine readily passes both the placental and fetal blood brain barriers, allowing maternal SDB-induced norepinephrine to influence the developing fetus (Piquer et al., 2017). Also recall that the locus coeruleus, the brain region responsible for norepinephrine production, isn't fully matured until the third postnatal week suggesting that maternal increases in norepinephrine caused by SDB may occur during a critical period of normally low or even no fetal norepinephrine exposure (Nakamura et al., 1987). Since norepinephrine decreases microglial motility and surveillance, we hypothesize that maternal SDB during pregnancy exposes fetal microglia to a bolus of norepinephrine during a critical period of development, leading to life-long alterations to their surveillance and therefore impaired pruning capabilities. In chapter 3, we will test whether GIH male offspring microglia have impaired surveillance through immunohistochemistry and fractal analysis.

Norepinephrine and Prenatal Stress

In addition to blunting microglial surveillance, norepinephrine is also linked to the stress response. In fact, a spike in norepinephrine is a direct precursor to increased corticosterone levels, the stress hormone in rodents (Joëls et al., 2011; Plotsky et al.,

1987; Sugama et al., 2019; Weidenfeld and Feldman, 1991), and increased norepinephrine is necessary for stress-induced behavioral responses in rodents (Lustberg et al., 2020; Sugama et al., 2019). Therefore, we hypothesize that maternal SDB during pregnancy may be a source of prenatal stress. Prenatal stress models display cognitive and behavioral impairments in adult offspring like those observed in our GIH rat model. We hypothesize that GIH is also a model of prenatal stress, and, in the appendix, we will test whether our IH exposure leads to increased corticosterone levels compared to normoxia.

Protecting Prenatal Microglia from Maternal Norepinephrine

We know that GIH induces cognitive and behavioral impairments in adult, male offspring, and increases in maternal norepinephrine may play a role in impaired offspring microglial pruning. To protect against these impairments, we sought to block norepinephrine's interaction with developing microglia. Norepinephrine blunts microglial surveillance specifically through ß2-adrenergic receptor activation, but microglial ß1- and ß2-adrenergic receptors appear to act through the same G_s-type pathway with knockdown of either receptor in microglia leading to more reactive and inflammatory CNS environments (Evans et al., 2020; Färber et al., 2005; Liu et al., 2019; Stowell et al., 2019; Tanaka et al., 2002). We chose to utilize the non-specific ß-adrenergic receptor antagonist propranolol to block norepinephrine activation of microglia to prevent any potential compensation through the ß1-adrenergic receptor.

An Introduction to Propranolol

Propranolol is a nonselective ß-adrenergic receptor antagonist, meaning it blocks both ß1- and ß2-adrenergic receptors. It is typically prescribed as an anti-hypertensive medication, but it is also used to treat anxiety, stress, and migraines (Alexander et al., 2007; Bergman et al., 2018; Hallstrom et al., 1981; Smits and Struyker-Boudier, 1979; Welch, 1994). Additionally, propranolol has been used to block stress-induced morphological changes in adult microglia, increasing their surveillance levels to those of non-stressed controls (Johnson et al., 2005; Sugama et al., 2019). In chapter 4, we will test whether prenatal propranolol treatment alongside GIH prevents the cognitive and behavioral impairments observed in adult male offspring.

Propranolol in Pregnancy

Propranolol is commonly prescribed during pregnancy as an anti-hypertensive. It makes up about 15% of all ß-blocker use among pregnant women and is one of the most prescribed nonselective ß-adrenergic antagonists among pregnant women (Bergman et al., 2018; Duan et al., 2017). While it is considered safe for use during pregnancy, the long-term consequences to offspring behavior and cognition are virtually unexplored (Duan et al., 2018). As propranolol can pass both the placental and blood-brain barriers (Botterblom et al., 1993; Erkkola et al., 1982; Watanabe et al., 1990; Wohleb et al., 2011), it has the potential to impact the developing fetus during critical periods of development.

Consequences of Propranolol During Pregnancy

Because of the time and expense required for long-term testing of offspring cognitive abilities, regulatory bodies often defer to immediate perinatal outcomes when testing drug safety during pregnancy. For example, both preterm birth and low birthweight are highly

correlated with cognitive and behavioral impairments later in life and are often used as perinatal checkpoints during drug safety assessments (García Ventura et al., 2022; Kim et al., 2022; Silva et al., 2022). Importantly, early testing of propranolol showed a dose-dependent teratogenicity. In rats, higher doses of 50-150mg/kg/day resulted in fetal growth restriction and reduced litter sizes while a dose of 25mg/kg/day in rats was identified as safe, and doses currently used in human pregnancy have no significant effect on birthweight (Duan et al., 2018; Schoenfeld et al., 1978).

For long-term testing, only two tests to date have studied offspring exposed to propranolol prenatally beyond the first postnatal week, and neither studied offspring anxiety or social interactions, both of which are targets for propranolol treatment. The first study observed increased locomotor activity in between the second and fourth postnatal weeks in rats treated with 10-20mg/kg/day propranolol (Speiser et al., 1991). The second study identified impaired maze-learning ability in adult female offspring exposed to both 20mg/kg/day propranolol prenatally and a second postnatal stressor suggesting the effects of prenatal propranolol exposure can appear into adulthood (Ryan and Pappas, 1990). Importantly, both studies utilized “safe” doses of propranolol below those that showed obvious signs of teratogenicity. In chapter 4, we will test whether prenatal propranolol exposure leads to cognitive or behavioral impairments in adult offspring using tests for social interaction, long-term memory, and anxiety.

Concluding Statements

Overall, this thesis seeks to explain the cognitive and behavioral deficits observed alongside increased dendritic spine density in GIH adult male offspring. We hypothesize microglia are altered during postnatal development leading to impaired pruning of

dendritic spines. Chapter 3 will test whether GIH influences postnatal microglial development in terms of microglial number, phagocytic capabilities, or morphology in a manner that could impair their pruning capabilities. Meanwhile, chapter 4 will test whether prenatal treatment with propranolol influences offspring anxiety, memory, or social interaction behaviors while also determining its effectiveness in preventing GIH-induced cognitive and social impairments. Overall, this thesis will advance our knowledge and understanding of the long-term consequences of maternal SDB during pregnancy on offspring development, while also identifying potential long-term consequences of the commonly prescribed prenatal drug propranolol.

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CHAPTER III

GESTATIONAL INTERMITTENT HYPOXIA ALTERS ADULT OFFSPRING MICROGLIAL MORPHOLOGY WITHOUT INFLUENCING EARLY POSTNATAL MICROGLIAL DEVELOPMENT OR PHAGOCYTIC ABILITY

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In preparation

AOK: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Data curation, Writing, Editing, Reviewing.
AMV: Investigation, Validation – collected tissues and assisted with immunohistochemistry validation
KMM: Methodology – assisted in troubleshooting fractal analyses and colocalization
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ABSTRACT

While it is well established that prenatal exposures such as maternal immune activation and hypertension are linked to long-term cognitive impairments, the mechanisms underlying them are not fully established. Microglia, the immune cells of the central nervous system, have been linked to cognitive impairments through improper pruning of dendritic spines. Microglia are especially vulnerable to prenatal exposures due to their cellular longevity and self-renewing nature perpetuating any early life alterations to their function. Maternal sleep disordered breathing (SDB) during pregnancy is one such prenatal exposure with links to both maternal inflammation and hypertension. Using a rat model of maternal SDB during pregnancy, a previous study identified impaired cognitive and behavioral endpoints alongside increased dendritic spine density in corresponding brain regions of maternal SDB-exposed offspring (Vanderplow et al., 2022). This study explored various functional endpoints in these offspring microglia and identified a decrease in overall microglial complexity, ramification, and branching suggesting a reduced surveillance phenotype in maternal SDB exposed offspring microglia. These findings have implications for neuropsychiatric disorders in which superfluous synaptic density drives the cognitive phenotypes.

INTRODUCTION

Microglia, CNS resident innate immune cells, contribute to brain development in early life by maintaining CNS homeostasis through synaptic pruning of excess dendritic spines (Paolicelli et al., 2011; Schafer et al., 2012; Stevens et al., 2007; Wake et al., 2009; Weinhard et al., 2018). Microglia originate from the embryonic yolk sac, and they colonize the developing mouse brain between embryonic days 8.5 (E8.5) and E13.5 (Ginhoux et

al., 2013). From this point on, they are a self-renewing population that rapidly proliferates over the first two postnatal weeks of life, from dense, activated microglia clusters classically referred to as “fountains of microglia” (Hagemeyer et al., 2017; Huang et al., 2018; Masuda et al., 2020). Additionally, microglia turnover rate is quite slow in the homeostatic brain with human microglia turnover occurring about once every 4.2 years (Réu et al., 2017). Because of their self-renewing nature combined with individual cellular longevity, fetal insults such as prenatal stress or maternal inflammation can result in long-lasting changes to microglial function, ultimately leading to cognitive and behavioral impairments that may not appear until much later in life (Baldy et al., 2018; Catale et al., 2020; Osborne et al., 2021; Xiao et al., 2021).

Sleep disordered breathing (SDB) during pregnancy is one such early life insult that often goes overlooked, affecting an estimated 14% of otherwise healthy pregnancies, and up to 40% of obese pregnancies (Johns et al., 2020). Recent studies in pregnant women with mild (5-10 apneas per hour) SDB show increased levels of inflammatory cytokines including TNFα and IL-1ß and trending increases in IL-6, cytokines that are hallmarks of maternal immune activation (Alonso-Fernández et al., 2021; Serednytskyy et al., 2022). In addition to inducing inflammation, SDB also increases sympathetic nervous system activity and serum norepinephrine levels in nonpregnant models (Gonzalez-Martin et al., 2009; Li et al., 2018; Xing and Pilowsky, 2010). A recent study in pregnant women with gestational diabetes mellitis and SDB showed increased norepinephrine metabolites in serum (Serednytskyy et al., 2022), suggesting similar increases in sympathetic nervous system activity among pregnant individuals as well. Although SDB is associated with negative short-term effects on the fetus including low birthweight and preterm birth, both

of which are associated with cognitive and behavioral impairments later in life, the long-term consequences of maternal SDB during pregnancy on offspring neural function are largely unexplored (Carnelio et al., 2017; Ding et al., 2014; García Ventura et al., 2022; Kim et al., 2022; Silva et al., 2022).

To begin to tackle this, we exposed pregnant rats to 15 episodes per hour of intermittent hypoxia during gestation (GIH) to model moderate severity SDB (Johnson et al., 2018). We found that adult male GIH offspring, not female, had significant cognitive and social behavioral deficits, and that these impairments were accompanied by increased neuronal dendritic spine density in the medial prefrontal cortex, a brain region important for these cognitive and behavioral functions (Vanderplow et al., 2022). Dendritic spines rapidly increase in density over the first several postnatal weeks, after which time they are pruned to reach normal adult levels (Huttenlocher, 1979; Nimchinsky et al., 2002). Deviations of normal dendritic spine density are observed in many neural disorders including autism spectrum disorder, schizophrenia, and Alzheimer's Disease as well as in offspring exposed to prenatal insults that result in maternal immune activation (Comer et al., 2020; Györffy et al., 2020; Hutsler and Zhang, 2010; Sellgren et al., 2019; Xiao et al., 2021).

Microglia prune excess dendritic spines during postnatal brain development (Comer et al., 2020; Datta et al., 2020; Paolicelli et al., 2011; Stevens et al., 2007). Dendritic spine pruning takes place primarily during periods of sleep when microglia are more active and surveillant (Choudhury et al., 2020; Yasumoto et al., 2021). Importantly, microglial surveillance state fluctuates with levels of endogenous norepinephrine with higher norepinephrine levels causing slower process motility and a smaller surveillance area during wakefulness (Liu et al., 2019; Stowell et al., 2019). Importantly, SDB increases

circulating norepinephrine levels which may also impact microglial surveillance and their ability to contact and prune dendritic spines during sleep, when norepinephrine levels are naturally low.

Several proteins are implicated in microglial pruning of synapses, including complement proteins such as C3 and C1q that bind synapses that need to be pruned (Datta et al., 2020; Schafer et al., 2012; Stevens et al., 2007; Wang et al., 2020; Werneburg et al., 2020), microglial receptors such as CD11b that recognize complement and phagocytic receptors like TREM2 and CD68 (Filipello et al., 2018; Parker et al., 2022; Wang et al., 2022; Weinhard et al., 2018), and other neuronal molecules like fractalkine (CX3CL1) whose precise role in microglial synaptic pruning is still being elucidated (Paolicelli et al., 2011). Because male GIH offspring have increased dendritic spine density, and microglia are important for synaptic pruning during early postnatal brain development, here we hypothesized that microglia from GIH offspring may have impaired ability to prune dendritic spines.

To address this hypothesis, we explored several possibilities whereby male GIH microglia may contribute to decreased dendritic spine pruning ability: (1) the male GIH offspring brain may have fewer microglia overall and thereby, less cells available to actively prune synapses, (2) male GIH microglia have impaired phagocytic capacity and are therefore unable to efficiently prune spines, and (3) microglia from male GIH offspring have a reduced ability to survey and contact dendritic spines that need to be pruned. To explore these endpoints, we utilized flow cytometry to quantify changes in microglial number, qRT-PCR to assess alterations in the expression of genes involved in phagocytosis and synaptic pruning, and immunohistochemistry to evaluate microglial morphology,

surveillance and colocalization with PSD95, a marker of dendritic spines. Pinpointing a functional change in male GIH microglia early in postnatal brain development will be an important first step towards elucidating mechanisms that contribute to the increased dendritic spine density identified in the male GIH offspring brain, and to eventually develop therapeutic interventions that will mitigate the effects of this maternal exposure on her male offspring.

METHODS

Animal Use/Ethics Statement

All animal experimental procedures were performed according to the NIH guidelines set forth in the Guide for the Care and Use of Laboratory Animals and were approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee.

Pregnant Rat Intermittent Hypoxia Exposure Paradigm

Timed pregnant Sprague-Dawley rats (gestational day 9) were obtained from either Charles River (Wilmington, MA) or Envigo (Indianapolis, IN) and housed in AAALAC-accredited facilities with 12-hour light/dark conditions. Food and water were administered *ad libitum.* Dams were exposed to 15 episodes per hour of intermittent hypoxia (GIH; 10.5% O_2/21% O_2) or intermittent normoxia (GNx; 21% O_2/21% O_2) for 8 hours per day from gestational days 10-21 during the light period as previously described (Johnson et al., 2018; Vanderplow et al., 2022). The exposure parameters were chosen to closely mimic the desaturation and reoxygenation periods experienced by humans with mild sleep apnea during pregnancy (Senaratna et al., 2017). Dams were removed from exposure before birth to avoid directly exposing offspring to hypoxia.

Flow Cytometry for $CD11b^{+}/CD45^{low}$ Cell Frequency

Single cell suspensions of whole forebrain (minus cerebellum, brainstem, and olfactory bulbs) tissue homogenates from rats ranging in age from P3 to P28 were processed as described previously with minor modifications to avoid disproportionate loss of cell types (Kiernan et al., 2019). Briefly, single cell suspensions were created by passing through 100μm filters in dissociation buffer (0.1mg/mL DNase and 0.001M EDTA in HBSS) and then blocked with a 2.5% BSA in 1X PBS solution at 4°C for 10 minutes prior to staining. Suspensions were then stained using the following mouse anti-rat antibodies: 1:200 CD11b/c – PE-Cy7 (OX-42, 562222, BD Biosciences RRID:AB_11154584), 1:200 CD45 – APC (OX1, 17-0461-82, Invitrogen RRID:AB_10717666). Cells were stained for 40minutes at 4°C in the dark with gentle shaking. $CD11b/c^{+}$, $CD45^{+low}$ cells were designated as microglia. Flow cytometry was performed by a BD Fortessa Flow Cytometer. Gating strategy is presented in figure 1A. FCS files were analyzed via FlowJo software v10.0.7r2.

RNA Isolation and qRT-PCR

Whole forebrain (minus cerebellum, brainstem, and olfactory bulbs) homogenates were taken from postnatal day 14 and 21 GNx/GIH rat offspring and processed for qRT-PCR as previously described (Vanderplow et al., 2022). Briefly, tissue homogenates were sonicated in Tri-Reagent (Sigma, St. Louis, MO) and total RNA was isolated per manufacturer's instructions with the addition of Glycoblue reagent (Invitrogen, Carlsbad, CA) to improve yields. A complementary DNA library was prepared from 1μg of total RNA using MMLV reverse transcriptase and an oligo dT and random primers cocktail (Promega, Madison, WI), and qRT-PCR performed as previously described (Kiernan et

al., 2019; Vanderplow et al., 2022) using PowerSYBR green PCR mix on a StepOnePlus (Thermofisher) system. All primers were tested for efficiency using serial dilutions, and results were normalized to 18S RNA levels using a standard curve as 18S is unaffected by hypoxia treatment. Primers CX3CR1, P2Y12, and TMEM119 are all used as microglia-specific genes while CD68, C1qA, Trem2, and TyroBP are implicated in microglial phagocytosis and pruning pathways.

Primers Used:

Gene	Forward Primer	Reverse Primer
18S	CGGGTGCTCTTAGCTGAGTGTCCC	CTCGGGCCTGCTTGAACAC
CD68	AAGCAGCACAGTGGACATTCT	GTGCAGGTGAATTGCTGGAGA
CX3CR1	TGCTCAGGACCTCACCATGC	CACCAGACCGAACGTGAAGA
C1qA	GGAGGCAGGAACATCATGGAGAC	GGAATTCCTGCAACCCCGTC
P2Y12	GAGGGCTTTGGCAACGAAAC	CACCTCCATGGTCCTGGTTAT
TMEM119	AGAAGGAAGGGGGAGGGC	ACAGAACCAGCTCTGGGAAAA
Trem2	ACTTCAGATCCTCACTGGACC	GGGTTGGTGTGTGAGAGTGT
TyroBP	TCCTGGTGCTTTCTGTTCCTT	AGGAACATTCGCATCCACTCTG

In Vitro Microglial Phagocytosis

To determine whether GIH microglia have phagocytic deficits that may lead to impaired pruning, whole brain tissue homogenates from male postnatal day 3 rats were cultured for 14 days before shaking to acquire microglia as previously described (Kiernan et al., 2019). Male rats were chosen as previously identified pruning and behavioral deficits are male-specific (Vanderplow et al., 2022). Briefly, cultures were prepared from postnatal day 3 pups and cultured at 37°C/5% CO_2 for 14 days. Cells were then shaken at 170rpm for 2 hours to acquire relatively pure microglia. Cells were plated at $5x10^5$ cells/well in 12-well plates and incubated overnight. Cells were then given fresh media with or without 2µl/mL FITC IgG-latex beads (400291, Cayman Chemical Company, Ann Arbor, MI) and

incubated for 2 hours. Cells were then incubated with 1:1000 eFluor780 (65-0865-14, Invitrogen) for 5 minutes at room temperature for live/dead labeling followed by 1:200 CD11b/c antibody (same as above) for 20minutes at 4°C in the dark and CD11b/c^{+} cells were designated as microglia. A quick (~15seconds) trypan blue quench was performed to quench external FITC bead expression. Flow cytometry was performed as above to determine the phagocytic ability of microglia as a percent of living CD11b/c^{+} cells containing FITC^{+} beads. Gating strategy is presented in figure 3A.

Ex Vivo Microglial Phagocytosis

Male rats were euthanized at postnatal day 28 and perfused with cold 1X PBS to remove circulating immune cells from central nervous system vasculature. Prefrontal cortex was isolated and dissociated into single cell suspensions via papain enzymatic digestion and filtered via 40μm filters. Myelin was removed using a 30% Percoll gradient to create a microglia-enriched cell suspension. Suspensions were then incubated in serum-free media containing 2μL/mL of either FITC-IgG latex beads or complement-opsonized yellow-green latex beads (L4530, Sigma, St. Louis, MO) for 2 hours. Opsonizing of beads was performed by pre-incubating in serum for at least 2 hours at 37°C in the dark. Cells were then labelled, external beads quenched, and flow cytometry was performed as above to determine the phagocytic ability of microglia as a percent of living CD11b/c^{+} cells containing FITC^{+} beads. Gating strategy is presented in Figure 3C.

Immunohistochemical Staining

Eight-week-old GNx and GIH male rats were transcardially perfused with PBS followed by 1.5% PFA for 15 minutes. Following perfusions, forebrains (minus cerebellum,

brainstem, and olfactory bulbs) were isolated and post-fixed for 2 hours in 1.5% PFA prior to coronal sectioning at 150μm on a vibratome. Sections were blocked while free-floating in 3% Normal Donkey Serum (017-000-121, Jackson Immunoresearch RRID:AB_2337258) for 1 hour at room temperature with gentle shaking. Sections were then incubated shaking overnight at room temperature using 1:1000 Iba-1 anti-rabbit (019-19741, FUJIFILM Wako). After three washes in 1X PBS, sections were incubated shaking for 3 hours at room temperature in the secondary antibody 1:500 donkey anti-rabbit Alexafluor488 (A21206, Invitrogen RRID:AB_2535792). After three more washes in 1X PBS, sections were mounted on slides using VECTASHIELD Hardset Antifade mounting medium with DAPI (H-1500-10, Vector Laboratories).

Microglial Fractal Analyses: To analyze microglial morphological complexity, images were taken using a Nikon A1RS laser scanning confocal microscope in 0.7μm z-steps using a x60 oil immersion objective at 1.5x digital zoom under consistent laser settings. Images were then processed using ImageJ FIJI SNP neuroanalysis plugin for fractal analyses. Images were acquired from the medial prefrontal cortex layers 2/3. N = 9-14 microglia per animal were chosen at random and averaged for that animal, n=4 animals per treatment.

Statistical Analyses:

Data were analyzed using GraphPad Prism 9.3.1. Data in figures are presented as Mean ± SEM with all datapoints shown. Outliers were removed using the ROUT test for detecting multiple outliers. For direct comparisons between two groups, two-tailed Students' t-test was used. For comparison involving more than two groups, a 2-way

ANOVA with Tukey test for multiple comparisons was performed. A P value ≤ 0.05 was used as the significance cutoff (* P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001).

RESULTS

GIH Does Not Alter Microglial Number in Postnatal Development

To test whether there were differences in microglial cell numbers over the course of postnatal brain development, we used flow cytometry to quantify the relative abundance of $CD11b^{+}/CD45^{low}$ cells in male and female GNX and GIH offspring. $CD11b/c^{+}$, $CD45^{low}$ cells were designated microglia (Figure 1A). We observed a roughly 8-fold increase in the first two weeks post-birth from P3 to P14, followed by a significant decline between the second, third, and fourth postnatal weeks (Figure 1D), recapitulating the microglial developmental timeline previously outlined in postnatal mice (Nikodemova et al., 2015). While we found a significant effect of timepoint on microglial number with P14 offspring having significantly more microglia than any other timepoint ($P < 0.0001$) (Figure 1D), there was no significant influence of GIH (P3, $p = 0.8190$; P14, $p = 0.7056$; P21, $p = 0.9188$; P28, $p = 0.8251$) nor of sex (P3, $p = 0.4083$; P14, $p = 0.5533$; P21, $p = 0.8325$; P28, $p = 0.6847$) on relative microglial abundance at any timepoint tested (Figure 2C).

GIH Does not Impact Microglial Number or Phagocytic Gene Expression

To complement microglial number assessments, we next measured changes in the expression of microglia-specific genes from whole forebrain tissue homogenates using qRT-PCR. We quantified the relative expression levels of P2Y12, CX3CR1, and TMEM119; homeostatic microglia-specific genes (Kenkhuis et al., 2022; McKinsey et al., 2020; Sharma et al., 2021; Walker et al., 2020). There were no significant changes

detected at either postnatal day 14 (P2Y12, p = 0.6061; TMEM119, p = 0.9750; CX3CR1, p = 0.5950) or 21 (P2Y12, p = 0.1329; TMEM119, p = 0.0.2342; CX3CR1, p = 0.0515) in the expression of any of these genes either based on sex or treatment (Figure 2A). Note that while the CX3CR1_P21 is trending, the trending difference is between GNx Male and GIH female groups. Consistent with the flow cytometry results above, these results suggest that neither GIH treatment nor sex alter homeostatic microglial number or gene expression at these time points.

To explore whether microglia in GIH male offspring have alterations in the expression of phagocytic genes that may influence their capacity to prune dendritic spines, we performed qRT-PCR on whole forebrain tissue homogenates for C1qA, CD68, TREM2, and TyroBP/DAP12; genes implicated in microglial phagocytosis. Peak synaptic pruning takes place in later postnatal development, so we measured the expression of these phagocytic-related genes at postnatal day 28, when synaptic pruning is increasing (Huttenlocher, 1979; Juraska and Drzewiecki, 2020). We found no significant impact of treatment or sex on the expression of any gene (C1qA, p = 0.4706; CD68, p = 0.6663; TREM2, p = 0.0691; TyroBP, p = 0.5876], suggesting that GIH microglia do not have alterations in their phagocytic ability (Figure 2B).

GIH Does Not Impair Microglial Phagocytic Ability *In Vitro* or *Ex Vivo*

To investigate potential differences in microglia phagocytic function in GIH microglia that may impact dendritic spine pruning, we quantified microglial uptake of FITC-labeled IgG latex beads in primary microglia derived from male GNx and GIH offspring. Flow cytometry was used to determine the % of live microglia that were positive for FITC over

a 2-hour incubation period. There were no differences between GNx and GIH on *in vitro* uptake of FITC-IgG latex beads ($p = 0.4906$) (Figure 3B).

To determine whether GIH alters microglial phagocytic activity at a developmental period more relevant to synaptic pruning, and in a brain region where increased synaptic density was previously identified (Vanderplow et al., 2022), we prepared microglia enriched cell suspensions from the postnatal day 28 rat prefrontal cortex (PFC) and cultured them acutely with FITC-IgG latex beads for 2 hours in serum-free medium to quantify the prevalence of $FITC^{+}/CD11b^{+}/CD45^{+/low}$ cells in the GNx and GIH offspring brain (Figure 3C). There were no differences between GNx and GIH microglial FITC bead uptake ($p = 0.4095$) (Figure 3D). Lastly, because several complement proteins including C3 and C1q have been identified to play key roles in identifying synapses for elimination during the pruning period, we tested complement-specific differences in microglial phagocytosis. Acutely isolated microglia-enriched cell suspensions from the postnatal day 28 GNx and GIH PFC, were cultured with complement-opsonized fluorescent latex beads for 2 hours, and the percentage of bead uptake was quantified via flow cytometry in $CD11b^{+}/CD45^{+/low}$ cells. No differences in complement opsonized bead uptake were noted between GNx and GIH microglia ($p = 0.6619$), again suggesting similar ability to phagocytose and eliminate complement between the treatments (Figure 3E).

GIH Male Microglia Exhibit Reduced Complexity

To elucidate potential changes to microglial surveillance in adulthood that may impact their ability to contact and remove excess spines, we labeled microglial cell bodies with Iba1 and imaged microglia of layer 2/3 of the PFC (Figure 4A). We performed fractal analyses using the SNT plug-in of ImageJ FIJI to trace microglial cell bodies and measure

overall microglial complexity (Figure 4B). GIH male microglia display fewer branches compared to their GNx counterparts ($p = 0.0187$) (Figure 4C). Additionally, GIH ramification index is significantly reduced compared to GNx microglia suggesting a more amoeboid morphology ($p = 0.0289$) (Figure 4D). Finally, the complexity index of GIH microglia is significantly lower than GNx microglia ($p = 0.0325$) (Figure 4E). These reductions branching and overall complexity suggest a less surveillant phenotype, which supports the third hypothesis that microglial pruning of dendritic synapses is impaired due to an inability to properly survey and contact spines in need of pruning.

DISCUSSION

This is the first study to investigate whether GIH alters offspring microglia and reduces their dendritic spine pruning capabilities. While GIH does not appear to influence the developmental timeline in terms of microglial number or phagocytic ability in early postnatal life, it alters microglial morphology in adulthood leading to less complexity in male GIH microglia with lower branching and less ramified phenotypes. These changes in microglial morphology could result in less volume being surveyed by the GIH male microglia and fewer microglia-spine contacts leading to the reduced pruning of dendritic spines observed in adult male GIH offspring.

Microglial Number Across Development

To determine whether GIH male offspring have fewer microglia, we have mapped the developmental timeline of microglia in the postnatal rat for the first time. Our results are consistent with the developmental timeline of microglia in the postnatal mouse previously outlined by Nikodemova et al., following the general trend of rapid proliferation from P3

to P14 and an equally rapid decline to meet healthy adult numbers of ~5-10% of total CNS cells by P28 (Ginhoux et al., 2013; Nikodemova et al., 2015). While the previous study by Nikodemova et al. utilized both male and female mice, they did not separate out sexes for analysis, and previous GIH offspring studies show male-specific deficits in cognitive and behavioral endpoints, highlighting the importance of separating out sexes for analysis (Vanderplow et al., 2022). We have shown that neither sex nor the prenatal insult of gestational intermittent hypoxia alters this developmental timeline as both GNx and GIH offspring display similar levels of relative microglial abundance regardless of sex.

The previous methodology utilized counts of pure, $CD11b^{+}$ isolated microglia relative to whole brain weights. Using flow cytometry as an alternative, we can compare the number of microglia to the total number of processed cells using both CD11b and $CD45^{+/low}$ staining to identify microglia more accurately in the CNS opposed to solely relying on CD11b labeling as peripheral macrophages also display $CD11b^{+}$. We further support our flow cytometry results by pairing it with relative gene expression levels of key microglial genes in forebrain homogenates at P14 and P21 showing no effect of sex or treatment. While the gene expression data alone does not rule out fewer cells having greater expression of these genes or vice versa, when combined with our flow cytometry data we can conclude that the differences in pruning are not due to changes in overall numbers of microglia.

Microglial Phagocytosis and Pruning of Synapses

Latex beads are commonly utilized to analyze basic phagocytic function of microglia (Filipello et al., 2018; Sellgren et al., 2019; Wang et al., 2020), but they have previously been utilized primarily in *in vitro* environments. One of the biggest issues with *in vitro*

cultured microglia is their stark difference from the *in vivo* environment having been removed, processed, and then cultured for days to weeks before phagocytic assay. By freshly isolating microglia and determining their phagocytic ability *ex vivo* within hours and with no serum exposure, we remove several of the confounds observed *in vitro,* as culturing primary microglia in the presence of serum is known to change their gene expression patterns and phagocytic abilities over time (Bohlen et al., 2017).

Our *ex vivo* methodology is better able to explore mechanism behind any observed changes in phagocytic ability when compared to immunohistochemistry of tissue slices since it can be manipulated during the phagocytosis process rather than requiring genetic manipulation or pharmacologic intervention in the *in vivo* animal. For example, by preincubating beads in serum to induce complement opsonization, we were able to determine whether there was a specific deficit in complement-mediated phagocytosis as earlier studies showed deposition of complement proteins C3, C4, and C1q determines which dendritic spines are removed (Comer et al., 2020; Paolicelli et al., 2011; Schafer et al., 2012). While we observed no change in GIH male microglial ability to phagocytose IgG-coated or complement-opsonized beads, it is possible that the impaired pruning mechanism is in a synapse-microglia specific interaction. However, our functional *ex vivo* results combined with no changes in gene expression of several pruning and phagocytic related genes provide evidence that GIH male offspring microglia do not have a general phagocytic deficit.

While early studies focused specifically on phagocytosis of postsynaptic dendritic spines and the role of complement, later studies explored alternative methods of spine removal. One such study observed microglial trogocytosis of pre-synaptic elements described as

partial phagocytosis of the synapse leaving presynaptic material behind rather than entire engulfment of post synaptic material previously shown (Comer et al., 2020; Paolicelli et al., 2011; Schafer et al., 2012; Weinhard et al., 2018). While they did not identify a signaling molecule or protein that directed trogocytosis, they found it was independent of C3 expression via a C3-knockout mouse line (Weinhard et al., 2018). Other studies have observed remodeling of synaptic elements by microglia where ~10% of contacts between a microglia process and spine head result in the spine disappearing even after the process withdraws independent of phagocytosis (Cheadle et al., 2020; Wake et al., 2009; Weinhard et al., 2018). Thus, while we do not see a functional phagocytic deficit in GIH male microglia, our observed morphological changes reducing overall surveillance volume may result in pruning deficits through fewer microglia-spine contacts.

Microglial Morphology and Surveillance

Microglia undergo constant changes in morphology and surveillance volume in response to stimuli, both endogenous and exogenous. For example, microglial surveillance state fluctuates with levels of endogenous norepinephrine, with higher norepinephrine levels blunting process motility and surveillance volume during wakefulness compared to sleep when norepinephrine is lower (Liu et al., 2019; Stowell et al., 2019). These fluctuations in surveillance state correlate nicely with evidence that increased pruning takes place during sleep when microglia are more active and surveillant which allows for more frequent microglia-spine contacts (Choudhury et al., 2020; Yasumoto et al., 2021). Additionally, norepinephrine has been shown to blunt the microglial chemotaxis response towards “find me” signals such as ATP in culture which could contribute to the reduced surveillance (Cserép et al., 2020; Davalos et al., 2005; Gyoneva and Traynelis, 2013). Importantly,

increased norepinephrine levels are seen in patients with SDB. We hypothesize that SDB during pregnancy may expose fetal microglia to higher-than-normal levels of norepinephrine, leading to long-term changes in microglial surveillance, and impairing their ability to find and contact excess dendritic spines later in life. This could explain the decreased complexity observed in GIH male microglia relative to controls if their response to find-me signals are being blunted, resulting in lower exploration of their environment. Further experiments exploring GIH microglial response to stimuli such as ATP gradients and norepinephrine are necessary to fully elucidate the impact of GIH on microglial surveillance.

Conclusions

In summary, through the combined use of flow cytometry and qRT-PCR, our data support the conclusion that GIH has no impact on microglial number or phagocytic ability during early postnatal development. Our IHC and fractal analyses show a decrease in GIH male microglial complexity and ramification, suggesting a decrease in microglial surveillance ability. Further studies into how prenatal norepinephrine exposure may influence postnatal microglial morphology are underway.

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Conflict of interest:

The authors declare no conflict of interest.

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FIGURES

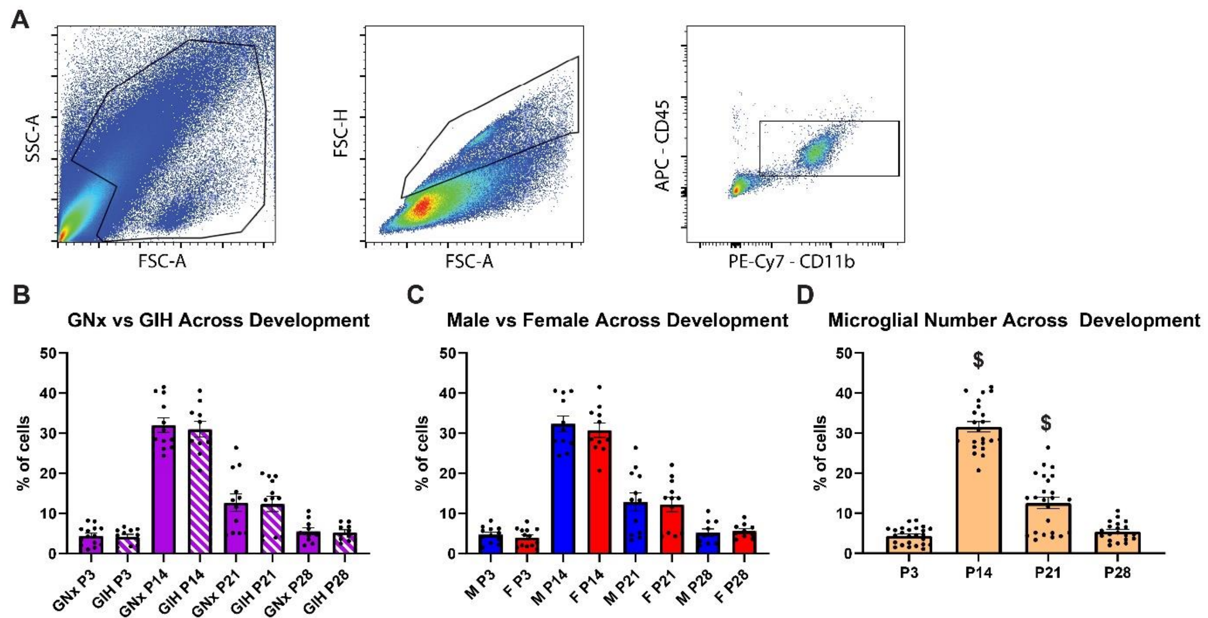

Figure 1: Relative microglial abundance changes across postnatal development but is unaffected by GIH. (A) Gating strategy used to identify microglial population via flow cytometry. All cells of appropriate size are then gated for single cells followed by $CD11b^{+}/CD45^{+/low}$ cells to designate microglia. **(B)** GIH treatment has no significant effect on microglial number at any age N=10-13 per timepoint [*P3* t (df, 33) = 0.03502, p = 0.9723; *P14* t (df, 20) =0.3490, p = 0.7308; *P21* t (df, 22) = 0.9506, p = 0.3521; *P28* t (df, 18) = 0.3137, p = 0.7573]. **(C)** Sex has no significant effect on microglial number at any age N=10-14 per timepoint [*P3* t (df, 23) = 0.8423, p = 0.4083; *P14* t (df, 20) =0.6030, p = 0.5533; *P21* t (df, 22) = 0.2140, p = 0.8325; *P28* t (df, 18) = 0.4127, p = 0.6847]. **(D)** Postnatal age has a significant effect on relative microglial abundance independent from sex or treatment. $ = p<0.0001 from all other age groups [Tukey post hoc (df, 61) p < 0.0001].

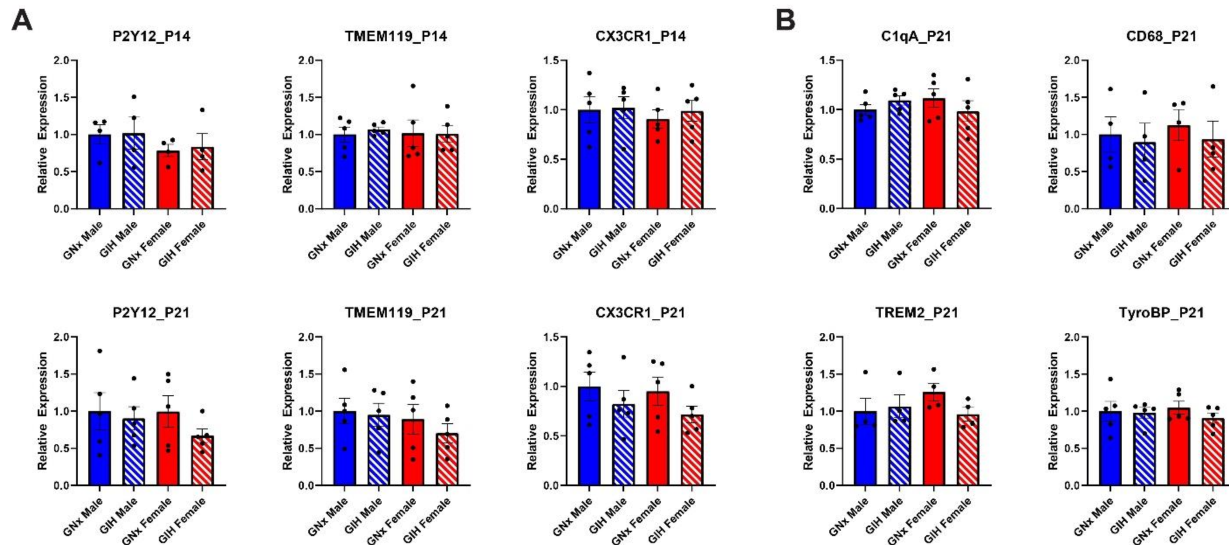

Figure 2: Relative gene expression of key microglial and phagocytic genes during postnatal development in unaffected by GIH. (A) Relative expression of microglia-specific genes in tissue homogenate samples are unaffected by sex or treatment at both P14 and P21 [*P2Y12_P14* Tukey post hoc (df, 9) p = 0.6061; *P2Y12_P21* Tukey post hoc (df, 12) p = 0.1329; *TMEM119_P14* Tukey post hoc (df, 12) p = 0.9750; *TMEM119_P21* Tukey post hoc (df, 12) p = 0.2342; *CX3CR1_P14* Tukey post hoc (df, 12) p = 0.5950; *CX3CR1_P21* Tukey post hoc (df, 12) p = 0.0515 – note this is due to a difference between GNx Male and GIH Female treatments, not a relevant interaction]. **(B)** Expression of several genes with a role in microglial phagocytosis are unaffected by sex or treatment. Results are displayed as the average fold change +/- SEM expressed relative to GNx males. N=4-5 animals per treatment across 3 or more litters. [*C1qA_P21* Tukey post hoc (df, 12) p = 0.4706; *CD68_P21* Tukey post hoc (df, 9) p = 0.6663; *TREM2_P21* Tukey post hoc (df, 9) p = 0.0691; *TyroBP_P21* Tukey post hoc (df, 12) p = 0.5876].

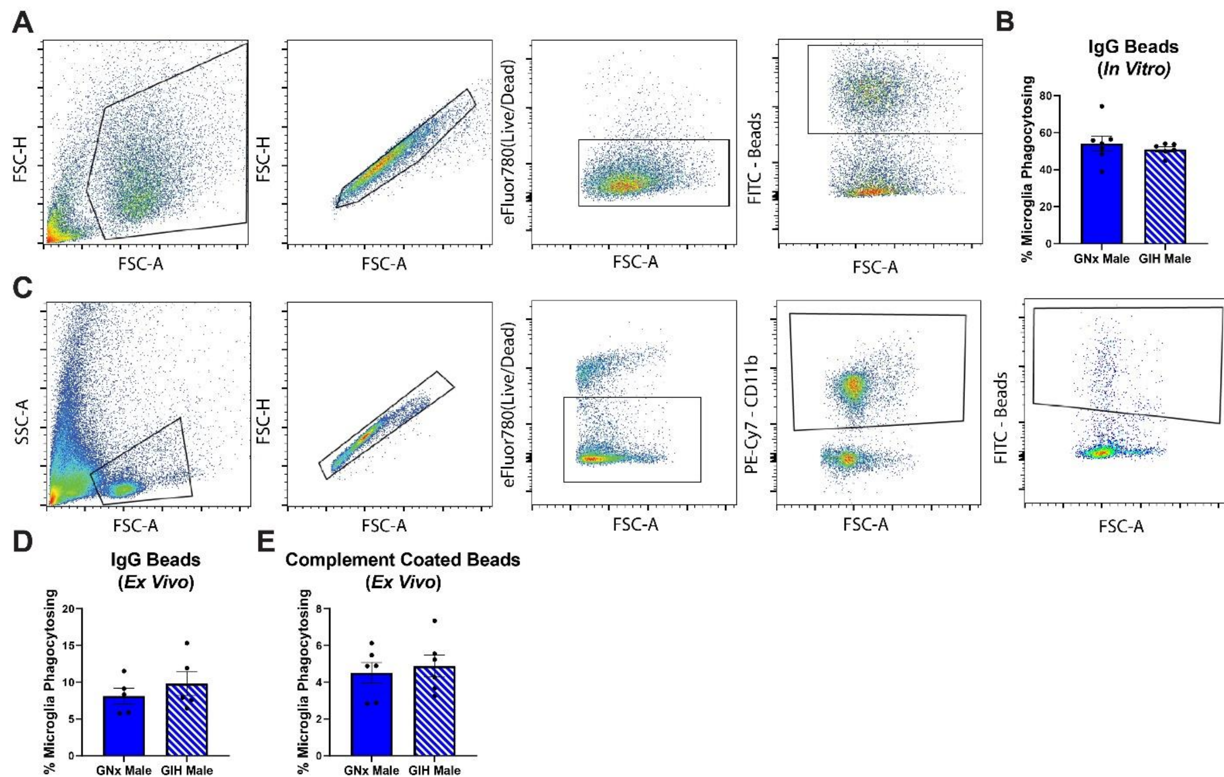

Figure 3: Postnatal microglial phagocytic ability is unchanged by GIH both *in vitro* and *ex vivo*. (A) Gating strategy used to identify phagocytic primary microglia when cultured for 14 days via flow cytometry. All cells of relevant size were gated for single, live cells that were FITC$^+$ and therefore phagocytic during the 2-hour incubation **(B)** Percent of total microglia positive for FITC$^+$ when primary cultured microglia were exposed to FITC$^+$ IgG-Latex beads for 2 hours. N=6-7 animals across 4 independent experiments. No significant impact of treatment [t (df, 11) = 0.7132, p = 0.4906]. **(C)** Gating strategy used to identify phagocytic microglia when collected from fresh tissue via flow cytometry. All cells of relevant size were gated for single, live cells. CD11b$^+$ cells were designated microglia and percent FITC$^+$ population were designated phagocytic. **(D)** Treatment has no significant effect on FITC$^+$ IgG-Latex bead uptake by freshly isolated microglia. N=5 animals per treatment across 4 independent experiments [t (df, 8) = 0.8702, p = 0.4095]. **(E)** Treatment has no significant effect on FITC$^+$ complement-coated latex bead uptake by freshly isolated microglia. N=6 animals per treatment across 5 independent experiments [t (df, 10) = 0.4506, p = 0.6619].

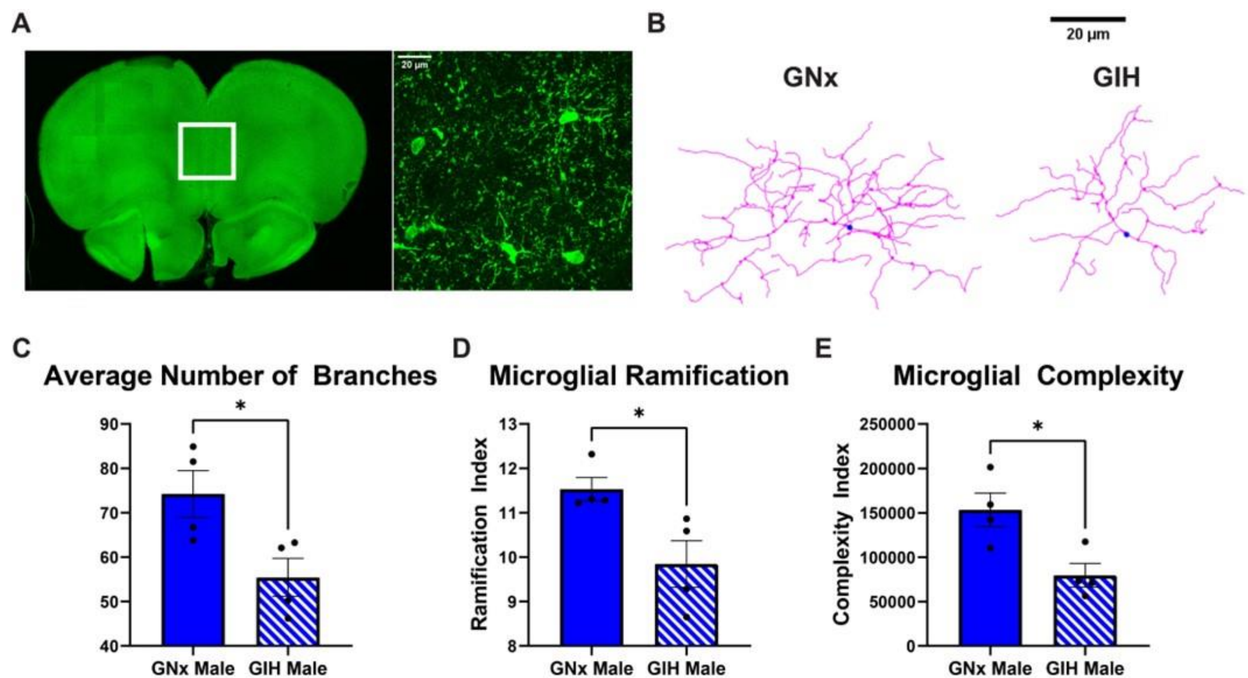

Figure 4: Microglial morphological complexity is reduced in GIH male offspring. (A) Microglia were imaged from the mPFC of rat forebrain sections. **(B)** Representative traces of GNx and GIH microglia generated by blind observer via SNT. Blue point denotes microglial soma. **(C)** Average number of branches per microglia was significantly increased by GIH treatment. N = 9-14 microglia averaged per animal; N = 4 animals per treatment [t (df, 6) = 3.196, p = 0.0187]. **(D)** Microglia Ramification Index was significantly decreased by GIH treatment. [t (df, 6) = 2.858, P = 0.0289]. **(E)** Relative microglia complexity was significantly decreased by GIH treatment. [t (df, 6) = 2.769, p = 0.0325].

CHAPTER IV

MATERNAL PROPRANOLOL ADMINISTRATION RESCUES SOCIAL APPROACH BEHAVIOR DEFICITS INDUCED BY GESTATIONAL INTERMITTENT HYPOXIA

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In preparation

AOK: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Data curation, Writing, Editing, Reviewing.
AMV: Methodology, Validation (Assisted in choosing and troubleshooting behavior tests, extensive work troubleshooting and validating spine analysis)
ID: Investigation (Performed behavior scoring alongside AOK)
TLB, Conceptualization, Funding acquisition
JJW, Conceptualization, Funding acquisition, Methodology, Editing, Reviewing

ABSTRACT

Maternal sleep disordered breathing (SDB) during pregnancy influences a substantial number of pregnancies and is associated with increased sympathetic nervous system activation. Using a model of maternal SDB where rats were exposed to intermittent hypoxia during the latter half of gestation (GIH), previous studies have identified long-term cognitive and behavioral impairments concomitant with increased dendritic spine density in related brain regions of GIH male offspring (Vanderplow et al., 2022). Microglia, the immune cells of the central nervous system, play a key developmental role through pruning of excess dendritic spines. Further investigation of GIH offspring microglia identified a reduced microglial surveillance phenotype and suggests an impaired ability to contact and remove excess dendritic spines. Recent literature has identified a role for the sympathetic hormone norepinephrine in modulating microglial surveillance phenotypes through activation of the ß2-adrenergic receptor. Concomitant with the GIH exposure period, we treated dams with 20mg/kg/day of propranolol, a nonspecific ß-adrenergic antagonist commonly used in human pregnancy to treat hypertension. In doing so, we selectively rescued the social behavior deficits previously identified in GIH male offspring while also identifying anxiety-like behaviors in offspring exposed to prenatal propranolol. These results suggest a role for prenatal ß-adrenergic receptor activity in long-term offspring cognitive endpoints while also identifying a possible long-term consequence of prenatal propranolol exposure when treating maternal hypertension.

INTRODUCTION

The maternal environment is critical to the development of a healthy offspring. The consequences of prenatal exposures to stress and inflammation on the developing offspring have been a topic of intense research for the past several decades (Ding et al., 2014; Maher et al., 2018; Tuovinen et al., 2014; Walsh et al., 2019). We are now aware of a host of high-risk morbidities during pregnancy that are associated with poor fetal outcomes including both maternal hypertension and sleep disordered breathing (SDB) (Ding et al., 2014). SDB affects an estimated 14%-40% of pregnancies, is frequently comorbid with hypertension, and is associated with increased sympathetic nervous system activity and elevated serum norepinephrine levels (Gonzalez-Martin et al., 2009; Grassi et al., 2015; Johns et al., 2020; Li et al., 2018; Sanapo et al., 2020; Takahashi et al., 2018; Torres et al., 2015). While hypertension has long been known to be associated with adverse cognitive outcomes in offspring, research into the long-term effects of maternal SDB is still in its early stages (Maher et al., 2018; Tuovinen et al., 2014; Vanderplow et al., 2022). Intermittent hypoxia is a hallmark of sleep disordered breathing, and our group has recently linked intermittent hypoxia during gestation (GIH) with cognitive and social behavioral impairments in male offspring, that is associated with an increase in dendritic spine density in associated brain regions (Vanderplow et al., 2022).

Microglia, resident immune cells in the central nervous system, play critical roles in brain development where they prune excess dendritic spines and remove cellular debris (Liu et al., 2022; Schafer et al., 2012). Recently, activation of the microglial ß2-adrenergic receptor by endogenous norepinephrine was shown to reduce microglial surveillance activity (Liu et al., 2019; Stowell et al., 2019), an observation that correlates with peak dendritic spine pruning occurring during sleep, when endogenous norepinephrine levels

are low (Choudhury et al., 2020). Since intermittent hypoxia increases circulating norepinephrine levels in individuals with SDB, we suggest that GIH may affect fetal microglia during a critical period of development, leading to long-term impairments in their surveillance and a reduced ability to identify, contact, and prune dendritic spines (Li et al., 2018; Takahashi et al., 2018). Further, given that the offspring cognitive impairments observed following GIH resemble those observed in offspring of hypertensive pregnancies, we hypothesized that GIH may act through a similar mechanism of enhanced adrenergic receptor signaling.

Propranolol is a nonselective ß-adrenergic receptor antagonist typically prescribed for hypertension, migraines, and anxiety. It is one of the most highly prescribed, nonselective ß-blockers used during pregnancy to treat hypertension (Alexander et al., 2007; Bergman et al., 2018; Beversdorf, 2020; Hallstrom et al., 1981; Welch, 1994). Propranolol readily crosses both the placental and blood brain barriers, and early rat studies exploring perinatal outcomes found that higher doses of 50-150mg/kg/day reduced litter size, increased caused embryonic resorption, and caused fetal growth restriction (Botterblom et al., 1993; Erkkola et al., 1982; Watanabe et al., 1990; Wohleb et al., 2011). However, doses lower than 25mg/kg/day resulted in otherwise normal birth outcomes in rats, consistent with its safe use in humans at lower doses (Duan et al., 2018; Ryan and Pappas, 1990; Schoenfeld et al., 1978; Speiser et al., 1991). Despite the prolific use of propranolol to treat hypertension in human pregnancy, there are virtually no long-term studies on offspring cognitive or behavioral outcomes.

In the prefrontal cortex, ß2-adrenergic receptor signaling is associated with both anxiety-like behaviors and reduced social interaction (Lei et al., 2022). Paradoxically, treating

adult rodents with ß-adrenergic receptor antagonists also impairs memory consolidation and spatial memory tasks (Evans et al., 2020; Gannon and Wang, 2019; Murchison et al., 2004), although few studies have investigated this after prenatal propranolol exposure. In the one study available (Ryan and Pappas, 1990), propranolol was given to pregnant dams, and her offspring were challenged with a stressful postnatal environment by withholding housing enrichment items after weaning. The authors found that adult female offspring exhibited significant spatial learning and memory deficits after this "second hit"; effects on other cognitive endpoints such as long-term memory, anxiety, and social behaviors following prenatal propranolol exposure have not yet been reported.

Thus, our study had 2 goals: to investigate the long-term consequences of prenatal propranolol exposure on offspring cognition and behavior, and to test whether propranolol could mitigate the detrimental effects of GIH on offspring cognitive deficits. We found that our prenatal dose of 20mg/kg/day propranolol had no adverse perinatal birth outcomes when combined with GIH exposure. We also found that while propranolol was able to rescue some GIH-induced offspring behavioral impairments, it did not mitigate them all. And lastly, to our surprise, we found that prenatal propranolol treatment induced some behavioral deficits on its own.

METHODS

Animal Use/Ethics statement

All animal experimental procedures were performed according to the NIH guidelines set forth in the Guide for the Care and Use of Laboratory Animals and were approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee.

Prenatal Hypoxia Exposure

Timed pregnant Sprague-Dawley rats (gestational day 9) were obtained from Charles River (Wilmington, MA) and housed in AAALAC-accredited facilities with 12-hour light/dark conditions. Food and water were administered *ad libitum.* Dams were exposed to intermittent hypoxia (GIH | 10.5% O_2/21% O_2) or intermittent normoxia (GNx | 21% O_2/21% O_2) for 8 hours per day during the light period as previously described from gestational days 10-21 (Johnson et al., 2018; Vanderplow et al., 2022). The exposure parameters were chosen to closely mimic the desaturation and reoxygenation periods experienced by humans with mild sleep apnea during pregnancy.

Prenatal Propranolol Exposure

Propranolol (20mg/kg) was administered daily from gestational days 10-21 alongside the gas exposure. To avoid unnecessary stress during gestation caused by restraint or injections, propranolol was administered through drinking water during the dark period (10mg/kg) and supplemented with 2 doses (5mg/kg each) mixed in Nutella (vehicle) consumed voluntarily during the light period spread ~4 hours apart to achieve a consistent dose. Drinking water was supplemented with 0.2% cherry Kool-Aid to mask flavoring. Control animals received 0.2% Kool-Aid supplemented drinking water and equal amounts of Nutella minus drug.

Behavior Testing

All offspring were behaviorally tested starting at 9 weeks of age, concluding with tissue harvests at 11 weeks of age. Age matching between GNx and GIH, Vehicle and Propranolol offspring was performed for all experiments. Testing was done on offspring

from a minimum of three litters per treatment group. Rats were assessed in multiple behavioral tasks including open field, novel object recognition, social interaction, and social novelty. All animal behaviors were videotaped with an overhead digital video camera. Behavioral scoring was performed while blinded to treatment conditions in each test.

Open Field test for Anxiety and Locomotion

Open field testing was performed as previously described (Vanderplow et al., 2022). Briefly, rats were placed in the open field chamber and allowed to explore for 10 minutes. Inner, middle, and outer zones were created using AnyMaze video tracking software, and time spent in each zone alongside distance traveled were automatically tracked. For more detail, reference the previously published study (Vanderplow et al., 2022).

Novel Object Recognition for Long-term Memory

Novel object recognition testing was performed as previously described (Vanderplow et al., 2022). Briefly, rats were habituated to the open field box for 10min each day both 48hours and 24 hours prior to testing. During the familiarization phase, rats were placed in the corner of the box facing away from two equally spaced identical objects (clear, smooth glass jars sealed with a white cap measuring 8.5cm tall, 4.0cm diameter). The rat was allowed to explore the objects for a combined total of 5 minutes. The testing phase occurred 24 hours later in a similar manner, but one of the identical objects was replaced by a novel object (ridged, pink translucent plastic bottle with a green translucent cap measuring 9.5cm tall, 4.8cm diameter). The rat was allowed to explore these two objects for a duration of 7 minutes. The recognition index was calculated as the portion of time

spent exploring the novel object divided by the total time exploring both objects. For quantification, video recordings were slowed down to 0.3x speed and time spent interacting with an object was quantified via Boris behavioral software (Friard and Gamba, 2016).

Social Recognition and Novelty (PFC/Amygdala dependent)

Social recognition and novelty testing was performed as previously described (Vanderplow et al., 2022). Briefly, rats were tested using a three-chamber box in three sessions. To habituate, rats were placed in the middle chamber of the empty three-chamber apparatus and allowed to freely explore for 10 minutes. For social recognition testing, the side chambers held an empty wire cylinder (nonsocial chamber) and a wire cylinder including an unfamiliar stimulus rat (social chamber). 24 hours post-habituation, rats were placed in the center chamber and allowed 7 minutes of exploration of the nonsocial and social chambers. Social recognition index was calculated as time directly investigating the stimulus rat divided by time spent investigating both cylinders. Immediately following the social recognition test, a novel unfamiliar stimulus rat was placed in the empty cylinder of the nonsocial chamber (hereby denoted novel chamber). The test rat was allowed an additional 7 minutes to explore the social and novel stimulus rats. Total exploration of each stimulus rat was then assessed for social novelty exploration. All stimulus rats were age and sex-matched to the test animals. For quantification, videos were analyzed while blinded to the observer at 0.3x speed using Boris behavioral software (Friard and Gamba, 2016).

Immunohistochemistry

Rats were transcardially perfused with phosphate-buffered saline (PBS) until clear, followed by 4% paraformaldehyde (PFA) in PBS for 10 minutes. Following perfusions, brains were removed and post-fixed in 4% PFA for 2 hours before moving to PBS. Slices were coronally sectioned at 150mM on a microtome and stored in PBS with 0.1% Sodium Azide before staining. Sections were blocked while free-floating in 3% Normal Donkey Serum (017-000-121, Jackson Immunoresearch RRID:AB_2337258) for 1 hour at room temperature with gentle shaking. Sections were then incubated shaking overnight at room temperature in primary antibody containing 1:1000 Iba-1 anti-rabbit (019-19741, FUJIFILM Wako). After three washes in 1X PBS, sections were incubated shaking for 3 hours at room temperature in the secondary antibody 1:500 donkey anti-rabbit Alexafluor488 (A21206, Invitrogen RRID:AB_2535792). After three additional washes in 1X PBS, sections were mounted on slides using VECTASHIELD Hardset Antifade mounting medium with DAPI (H-1500-10, Vector Laboratories).

Microglial Fractal Analyses: To analyze microglial morphological complexity, images were taken using a Nikon A1RS laser scanning confocal microscope in 0.7μm z-steps using a x60 oil immersion objective at 1.5x digital zoom under consistent settings. Images were then processed using ImageJ FIJI SNP neuroanalysis plugin for fractal analyses. Images were acquired from the medial prefrontal cortex layers 2/3. Ten microglia per animal were chosen at random and averaged for that animal, n=4 animals per treatment.

Statistical Analysis

Data were analyzed using GraphPad Prism 9.3.1. Data in figures are presented as Mean ± SEM with all datapoints shown. Outliers were removed using the ROUT test for

detecting multiple outliers. For direct comparisons between two groups, two-tailed Students' t-test was used. For comparison involving more than two groups, a 2-way ANOVA with Tukey test for multiple comparisons was performed. A P value ≤ 0.05 was used as the significance cutoff (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

RESULTS

Neither Propranolol, GIH, Nor Their Combined Treatment Impacts Perinatal Outcomes

To confirm that a dose of 20mg/kg/day does not induce negative perinatal outcomes, litter distributions and maternal weights throughout gestation were recorded. Average daily propranolol dose varied slightly due to voluntary consumption, but variation was insignificant between treatments ($p = 0.9612$) (Figure 1A). Regardless of treatment, all dams exhibited roughly equivalent changes in body weight gain throughout pregnancy, suggesting that neither prenatal propranolol nor gestational intermittent hypoxia treatment have an overt effect on fetal growth rates at these doses ($p = 0.720$) (Figure 1B). This is in line with previously published studies utilizing <25mg/kg/day propranolol showing no significant effects to perinatal outcomes (Ryan and Pappas, 1990; Schoenfeld et al., 1978; Speiser et al., 1991). Additionally, while some prenatal exposures including higher doses of propranolol have been known to alter litter size or distribution, neither treatment had any effect at these doses ($p = 0.3140$) (Figure 1C) (Schoenfeld et al., 1978).

Prenatal Propranolol and GIH Exposures Induce Anxiety Phenotypes in Adult Male, but Not Female Offspring:

To determine whether prenatal propranolol or GIH impact adult offspring anxiety phenotypes, we measured their activity level in the open field test. The open field test is commonly used to test anxiety and mobility deficits in adult rats. Rats naturally spend the majority of their time exploring the outer edges rather than the middle and inner zones where they are more exposed and vulnerable. Because of this, the relative amount of time spent exploring these middle and inner zones can be used as a test of general anxiety for the subjects with more anxious subjects spending significantly less time in the center. While neither prenatal propranolol nor GIH exposure alone impact overall distance travelled, the combined exposure significantly reduces total distance traveled in adult male offspring ($p = 0.0239$) (Figure 2A). This is due to a lower average speed of travel ($p = 0.0314$) and not less time spent mobile ($p = 0.3735$) (Figures 2B-C). This suggests a slower ambulatory phenotype in these animals rather than a reduced motivation to initiate exploration. All three treatments result in adult male offspring spending significantly less time in the middle (Propranolol $p = 0.0010$; GIH $p = 0.0010$; Combined $p = 0.0007$) and inner zones (Propranolol $p = 0.0007$; GIH $p = 0.0308$; Combined $p = 0.0050$), suggesting an increase in anxiety-like phenotypes in males from these treatments as they spend more time in the “safer” outer zone (Figures 2D-E). While propranolol and combined exposures have no influence on latency for first exit in adult male offspring, GIH exposure trends towards a later exit ($p = 0.0618$) (Figure 2F). Despite the changes in time spent within the inner and middle zones in adult male offspring, neither middle ($p = 0.6697$) nor inner ($p = 0.4029$) zone visit duration is significantly affected by any treatment suggesting fewer visits to these zones rather than less time spent in a zone per individual visit (Figure 2G-H).

While there is an increase in anxiety in the adult male offspring, the adult females are minimally impacted by each exposure. They again exhibit the reduced distance travelled in the combined exposure group (p = 0.0337) as a result of lower average speed (p =0.0304), but not less time spent mobile (p = 0.5184) (Figures 3A-C). This again suggests a slower ambulatory phenotype in these animals rather than a reduced motivation to initiate exploration. Unlike the adult males, the adult female offspring spend an equal amount of time in middle (p = 0.0915) and inner (p = 0.1978) zones regardless of treatment (Figures 3D-E) suggesting that the anxiety phenotype is male-specific. Despite spending an equal amount of total time in the middle and inner zones, the prenatal propranolol group exhibited significantly increased latency to first exit from the outer zone (p = 0.0150) (Figure 3F). While the total time spent in the middle zone did not differ, the average visit duration was increased in combined exposure group relative to the propranolol treated group (p = 0.0322) (Figure 3G). Additionally, the average visit duration to the inner zones was significantly increased by GIH (p = 0.0250) and combined (p = 0.0025) exposures, but not prenatal propranolol alone suggesting this effect is due to GIH exposure and independent of propranolol (Figure 3H).

Prenatal Propranolol has no effect on Novel Object Long-term Memory

To explore the effects of prenatal exposure to propranolol and GIH on long-term memory, we tested adult offspring using the novel object recognition test. The novel object recognition test allows us to study both long-term memory through the ability to recognize familiar versus novel stimuli as well as the motivation to explore novelty through the total time spent exploring objects. We observed no effect of prenatal propranolol nor GIH on overall motivation to explore novelty, as the total amount of time spent exploring the

objects during both the familiarization (Males, p = 0.7334; Females, p = 0.3700) and recognition (Males, p = 0.4425; Females, p = 0.1527) days was unaffected by treatment in either sex (Figure 4A-B & 4D-E). There was, however, a noticeable effect of GIH on adult male long-term memory as both GIH alone and the combined treatment but not propranolol alone had significantly impaired recognition indexes (Propranolol, p = 0.7089; GIH, p = 0.0041; Combined, p = 0.0331) (Figure 4C). Unlike the males, no treatment had a significant effect on adult female offspring (p = 0.0947) suggesting the long-term memory deficits are male offspring specific (Figure 4F).

Prenatal Propranolol Rescues GIH-induced Social Approach Deficits in Adult Male Offspring:

To assess the effects of prenatal propranolol and GIH on adult offspring social behavior, we utilized the 3-chamber social interaction test. This test first assesses the animal's social approach behavior, followed by their tendency to interact with a novel or familiar conspecific. Interestingly, prenatal propranolol exposure increased total interaction time with the test chambers relative to all other treatment groups in adult male offspring, suggesting an increased tendency towards exploration that is sex-specific (p <0.0001) (Figure 5A). GIH treatment alone has previously been shown to reduce social approach behavior in males, a result that has been recapitulated here (p = 0.0028). Interestingly, prenatal propranolol has no effect on social approach on its own (p = 0.3069), but when combined with GIH, it rescues the previously seen deficits, restoring adult male offspring to control levels of social approach (p = 0.7927) (Figure 5B). When assessed for social novelty, both GIH and combined treatments but not propranolol alone significantly reduced total interaction time suggesting an effect of GIH that is not rescued by

propranolol (Propranolol p = 0.6778; GIH p = 0.0037; Combined p = 0.0008). Despite this reduced total exploration time in GIH offspring, no treatment significantly altered social novelty preference in adult male offspring (p = 0.8884). While the males display impairments to social approach behavior in GIH cohorts that is rescued by propranolol, adult female offspring display no treatment-specific effects to total interaction time, social approach index, novelty interaction time, or social novelty preference (Social Interaction, p = 0.3327; Social Index, p = 0.5149; Novelty Interaction, p = 0.9906; Novelty Preference, p = 0.5154).

DISCUSSION

This is the first study to date to explore the long-term consequences of prenatal propranolol with and without GIH exposure in terms of anxiety, long-term memory, and social interaction behaviors. We have identified: 1) increased anxiety phenotypes in male, but not female offspring from both propranolol- and GIH-treated dams, 2) an important reversal by maternal propranolol treatment of GIH-induced social approach behavioral impairments in male offspring, and 3) impaired long-term memory in GIH adult male offspring that is not corrected by prenatal propranolol exposure.

While we hypothesize a role for the microglial ß2-adrenergic receptor in the GIH-induced cognitive and behavioral deficits, the ß1-adrenergic receptor may also play a role. Microglia express both ß1- and ß2-adrenergic receptors, both of which are G_s-coupled and result in increased cAMP production (Motiejunaite et al., 2021; Sugama et al., 2019). While norepinephrine has been shown to activate the ß2-adrenergic receptor in microglia, it typically has a higher affinity for the ß1-adrenergic receptor (Motiejunaite et al., 2021).

Although norepinephrine pretreatment attenuates LPS-induced inflammation, the effects of norepinephrine can be blocked by both ß1- and ß2-receptor antagonists, suggesting some overlap in their function. Additionally, propranolol prevented stress-induced activation of microglia in adult rats bringing their surveillance activities back to control levels (Sugama et al., 2019). We therefore chose to use the non-selective ß-blocker propranolol in our studies here. Further, since norepinephrine release occurs early in the HPA axis stress response, and it is elevated in humans with sleep apnea (Pinto et al., 2013), we hypothesized that propranolol would be a reasonable candidate to block the negative cognitive outcomes in GIH offspring. Lastly, propranolol is one of the most commonly prescribed non-selective ß-blockers in human pregnancy, making its study more clinically relevant.

No negative effects on typical perinatal outcomes including maternal weight gain, litter size, or sex distribution were observed using the 20 mg/kg/day maternal dose of propranolol used here. This is consistent with available literature indicating that doses of <25mg/kg/day are considered safe in pregnant rodents (Schoenfeld et al., 1978). Therefore, the negative cognitive outcomes that we report here in adult offspring suggest that these “safe” propranolol doses may indeed induce previously unrecognized long-term deficits.

Consistent with our previous study (Vanderplow et al., 2022), we found that GIH male but not female offspring have impaired social approach behavior in adulthood. Importantly, in this study, we found that prenatal propranolol treatment of GIH-exposed dams could rescue these social deficits. However, the GIH-induced decrease in novel object recognition that is observed in male offspring was not reversed by prenatal propranolol

exposure, suggesting that the effects of prenatal propranolol are perhaps specific for different brain regions and/or circuits. We hypothesize that prenatal propranolol treatment prevents activation of fetal microglia during a key period of brain development by blocking aberrant activation of fetal ß2-adrenergic receptors on microglia by maternal norepinephrine. Ongoing studies are investigating the effects of propranolol on offspring microglial morphology and process complexity. When given prior to the stressor, propranolol treatment prevents stress-induced morphological changes to adult microglia, keeping them in a ramified state (Di Martino et al., 2020; Sugama et al., 2019). Many prenatal challenges alter offspring microglial morphology towards a more amoeboid phenotype later in life (Mosser et al., 2017; Murray et al., 2019); therefore, we hypothesize that propranolol may mitigate some of these prenatal exposures' effects on offspring microglial morphology long-term.

Our novel observation here that adult offspring of prenatal propranolol-treated dams have male-specific anxiety-like behaviors was unexpected because propranolol is commonly prescribed as an antianxiety medication (Hallstrom et al., 1981; Wohleb et al., 2011). However, in the PFC, the ß2-adrenergic receptor is expressed on excitatory neurons where its activation induces anxiety-like behaviors in adult mice. In rodents, the prefrontal cortex develops during the latter half of pregnancy (Arain et al., 2013; Lei et al., 2022; Rice and Barone, 2000) and coincides with the prenatal propranolol treatments in our studies, which began on gestational day 10. Thus, it is possible that exposure to propranolol and GIH during this critical period of development altered ß2-adrenergic signaling in fetal PFC neurons, resulting in the increased anxiety-like behaviors observed

in prenatal propranolol treated offspring. Additional studies are necessary to fully investigate this possibility.

The anxiety-like behaviors we observed in GIH offspring in this study differ from the results of our previous study in which GIH offspring did not display an increased anxiety-like phenotype (Vanderplow et al., 2022). However, it is important to note that our previous study did not include daily interaction and handling/weighing of the pregnant GIH dams to deliver the propranolol or vehicle treatments during their pregnancy; it is possible that this daily handling resulted in a “second hit” of increased maternal stress and perhaps worsened the GIH phenotype. In addition to the anxiety-like phenotype in these GIH offspring, we also observed a decrease in the distance travelled in the open-field test due to a decrease in their average speed though their total time mobile was unchanged in the GIH+propranolol-treated offspring of both sexes. One potential interpretation of this result is that the offspring exhibited a more cautious exploration phenotype while their overall motivation to explore was unaffected.

Significance

In conclusion, while prenatal propranolol exposure may significantly increase anxiety-like behaviors in adult male offspring, it appears not to affect long-term memory as assessed by novel object recognition. GIH-induced social approach deficits, on the other hand, were rescued by prenatal propranolol treatment suggesting that propranolol may be selectively protective against certain behavioral impairments in adulthood. Based on the protective effects of propranolol in rescuing GIH-induced social deficits, we hypothesize a role for aberrant ß2-adrenergic receptor activation during male GIH offspring fetal brain development that is evident in adulthood. Future studies will further explore its role in

GIH-induced microglial morphological changes. Our results suggest that prenatal propranolol exposure alone may have a significant negative impact on offspring anxiety levels in adulthood. Therefore, it may be prudent to prescribe more ß1-selective adrenergic receptor antagonists such as metoprolol or atenolol when attempting to control hypertension during the latter stages of pregnancy. By selecting ß1-selective ß-blockers, potential interactions with the PFC neuronal ß2-adrenergic receptors whose inhibition is associated with anxiety (Bergman et al., 2018; Lei et al., 2022) would be avoided.

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Conflict of Interest

None of the authors have competing interests, including no conflict of interest, for this work.

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FIGURES

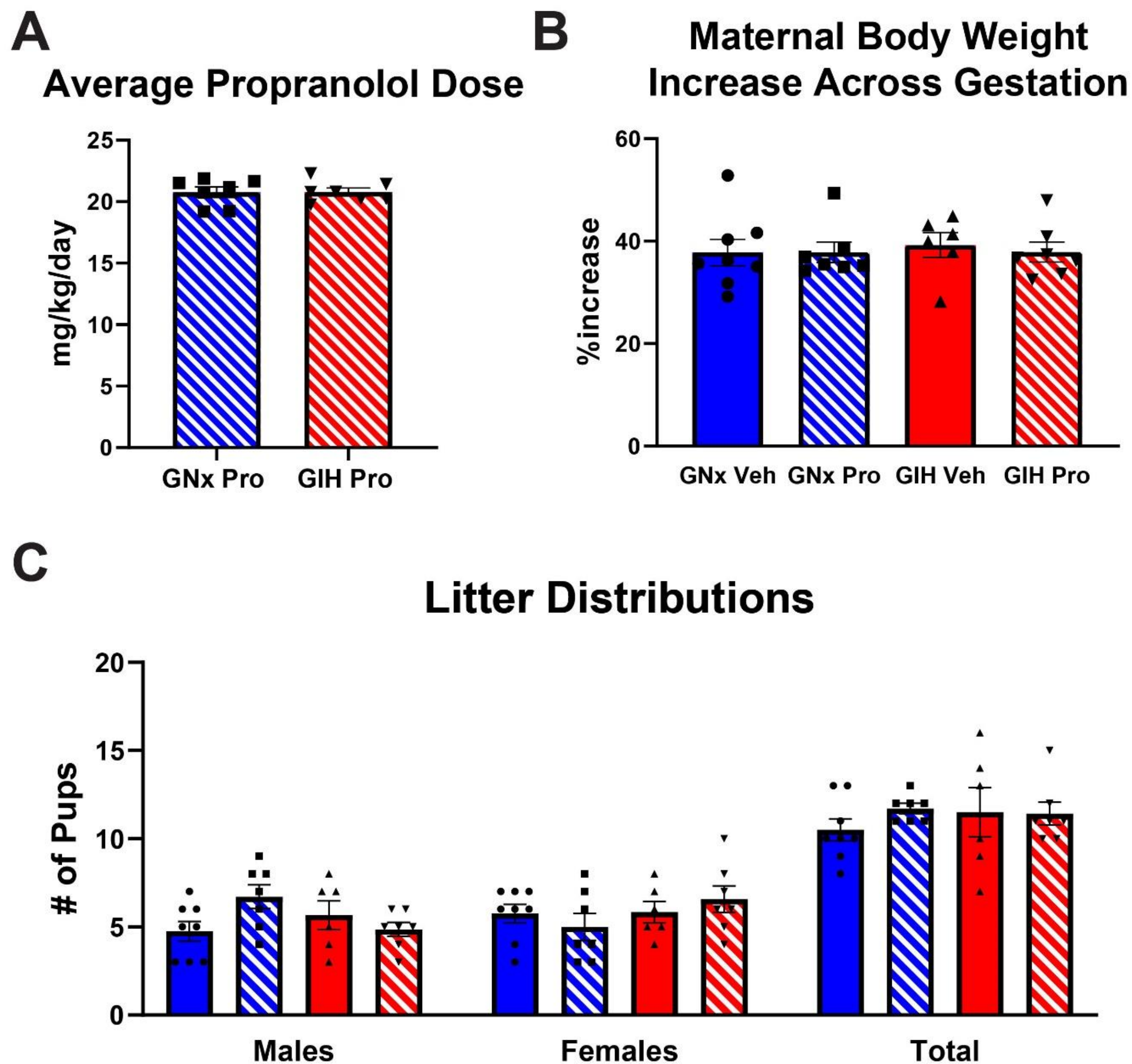

Figure 1: Perinatal outcomes are unchanged in prenatal propranolol and GIH treated offspring. (A) The total daily dose of propranolol given to GNx and GIH dams through wake period drinking water and sleep period voluntary consumption did not significantly differ [t (df, 12) = 0.04974, p = 0.9612] **(B)** Maternal body weight gain was unaffected by either propranolol or intermittent hypoxia treatments [Tukey post hoc (df, 17) p = 0.72.] **(C)** Neither prenatal propranolol or GIH treatments influenced litter size or sex distribution. [Tukey post hoc (df, 17) male p = 0.0813; female p = 0.3981; total p = 0.3140]. N = 6-8 litters per treatment.

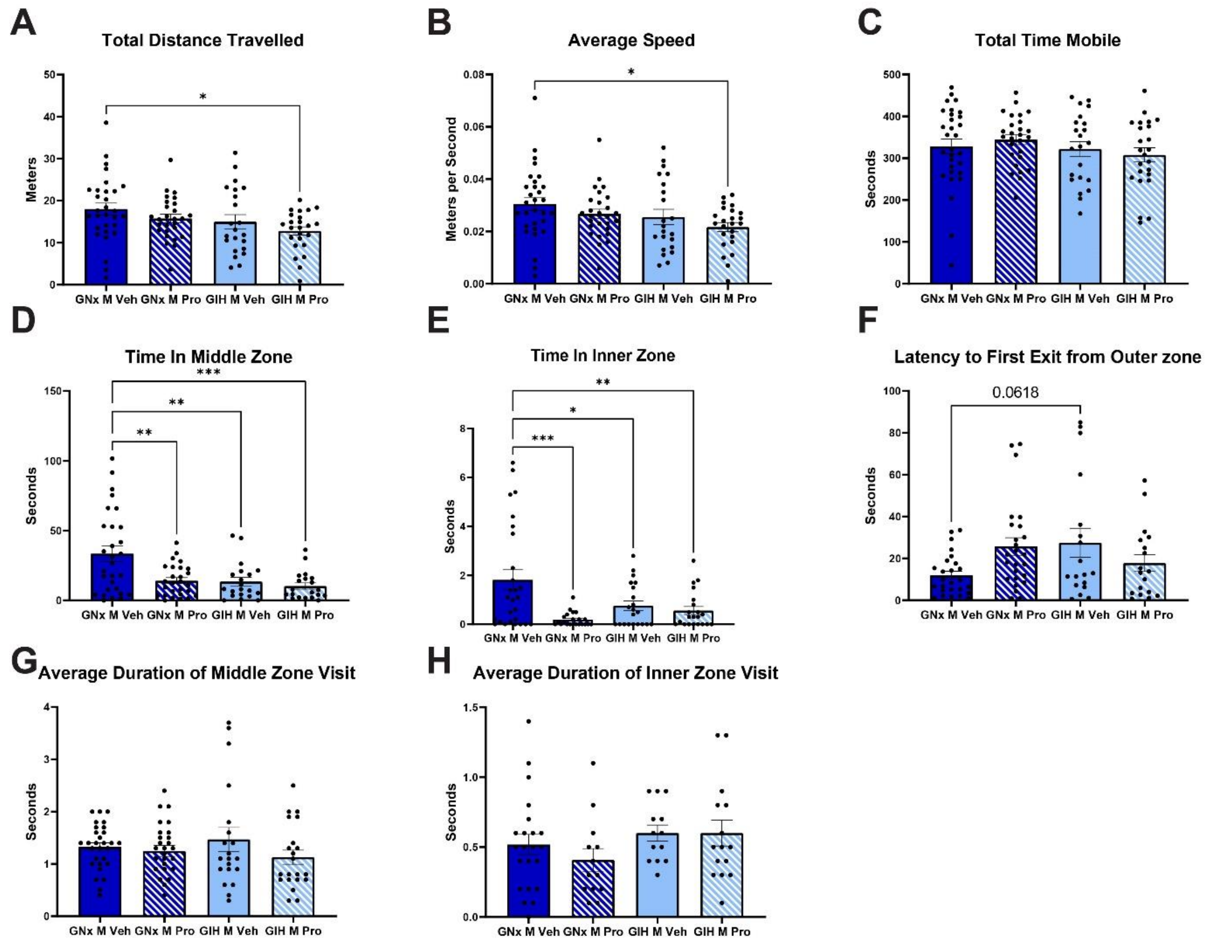

Figure 2: Adult *male* offspring display anxiety-like behaviors in open field testing. **(A)** Total distance travelled is reduced by combined treatment of prenatal propranolol and GIH, but not by either treatment alone [Tukey post hoc (df, 70) Propranolol p = 0.5922; GIH p = 0.2922; Combined p = 0.0239]. **(B)** Average speed traveled is unaffected by either treatment alone, but reduced by their combined treatment [Tukey post hoc (df, 70) Propranolol p = 0.6166; GIH p = 0.3229; Combined p = 0.0314]. **(C)** Total time spent mobile was unaffected regardless of treatment [Tukey post hoc (df, 70) p = 0.3735]. **(D)** Time spent in middle zone was reduced by both GIH and prenatal propranolol exposure [Tukey post hoc (df, 70) Propranolol p = 0.0010; GIH p = 0.0010; Combined p = 0.0007]. **(E)** Each treatment reduced time spent in inner zone [Tukey post hoc (df, 70) Propranolol p = 0.0007; GIH p = 0.0308; Combined p = 0.0050]. **(F)** Latency until first exploration out of starting zone was not significantly affected by treatment [Tukey post hoc (df, 70) p = 0.0575]. **(G)** Average middle zone visit duration was unaffected by treatment [Tukey post hoc (df, 70) p = 0.6697]. **(H)** Average duration of visit to inner zone is independent of treatment [Tukey post hoc (df, 70) p = 0.4029]. N=22-30 animals per treatment.

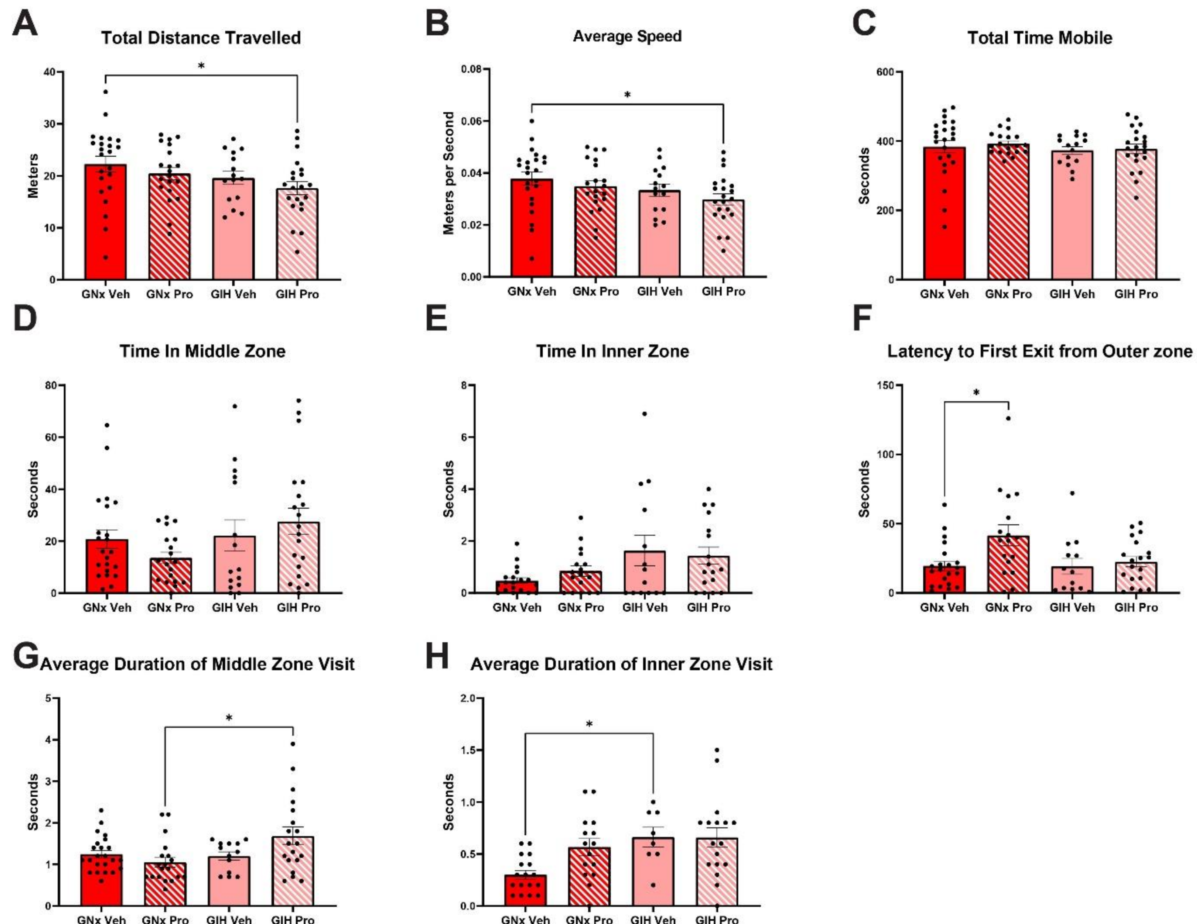

Figure 3: Adult *female* offspring do not display anxiety-like behaviors in open field testing. (A) Total distance travelled is reduced by combined treatment of prenatal propranolol and GIH, but not by either treatment alone [Tukey post hoc (df, 53) Propranolol p = 0.4922; GIH p = 0.3181; Combined p = 0.0337]. **(B)** Average speed traveled is unaffected by either treatment alone, but reduced by their combined treatment [Tukey post hoc (df, 53) Propranolol p = 0.5139; GIH p = 0.2978, Combined p = 0.0304]. **(C)** Total time spent mobile was unaffected regardless of treatment [Tukey post hoc (df, 53) p = 0.5184]. **(D)** Treatment had no effect on time spent in middle [Tukey post hoc (df, 53) p = 0.0915] or **(E)** inner zones [Tukey post hoc (df, 53) p = 0.1978]. **(F)** Latency until first exploration out of starting zone was significantly impaired in prenatal propranolol offspring, but not in GIH or combined treatments [Tukey post hoc (df, 53) p = 0.0150]. **(G)** Combined exposure significantly increased average middle zone visit duration compared to propranolol alone [Tukey post hoc (df, 53) p = 0.0322]. **(H)** Average duration of visit to inner zone was significantly increased by both GIH alone and combination treatments [Tukey post hoc (df, 53) Propranolol p = 0.3579; GIH p = 0.0250; Combined p = 0.0025]. N=8-17 animals per treatment.

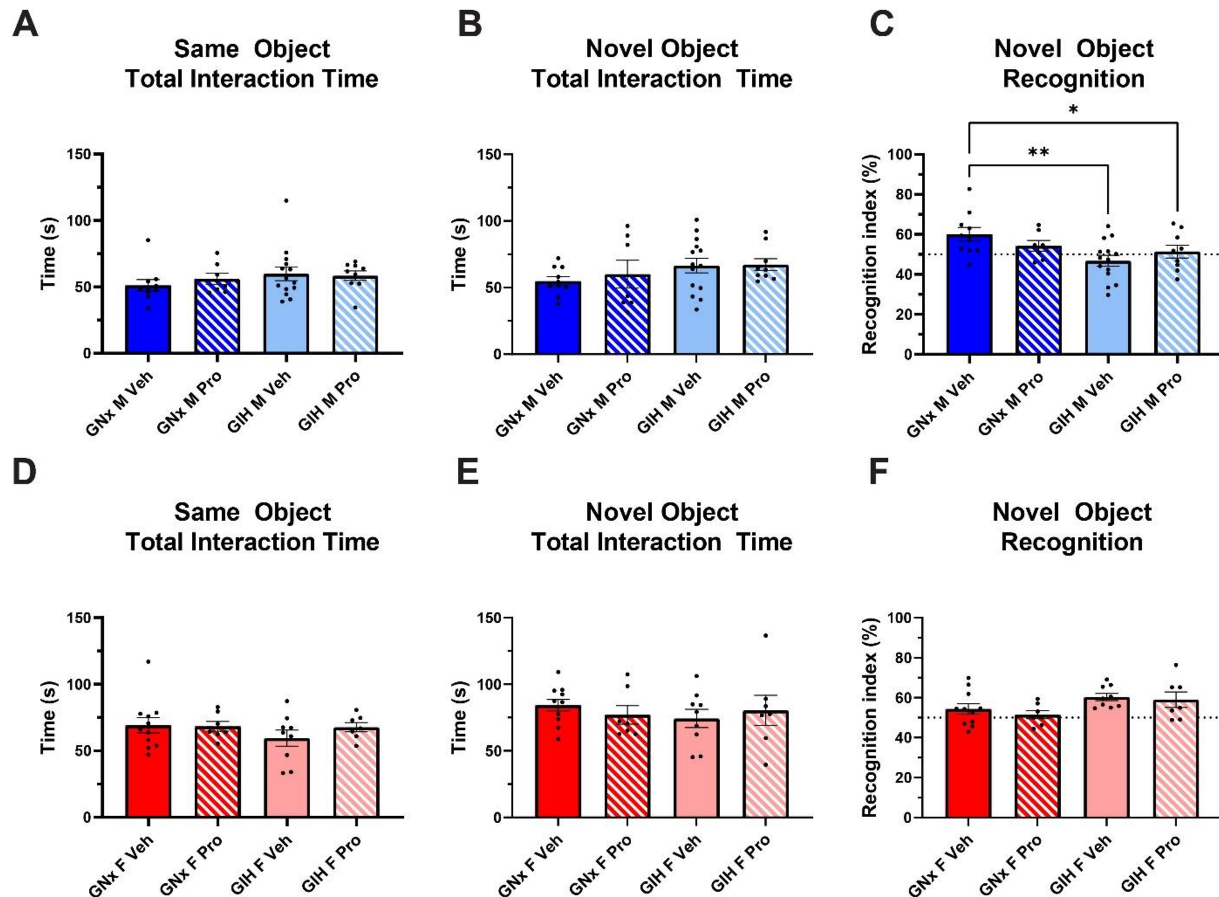

Figure 4: GIH male, but not female, offspring exhibit long-term memory impairments in novel object recognition testing. Total interaction time of adult *male* offspring with both objects is unaffected by treatment during **(A)** same object familiarization phase [Tukey post hoc (df, 21) p = 0.7334] and **(B)** novel object recognition phase [Tukey post hoc (df, 21) p = 0.4425]. **(C)** Novel object recognition of adult *male* offspring is significantly impaired in both GIH and combined treatments, but not with propranolol alone [Tukey post hoc (df, 21) Propranolol p = 0.7089; GIH p = 0.0041; Combined p = 0.0331] n=7-14 animals per treatment. Total interaction time of adult *female* offspring with both objects is unaffected by treatment during **(D)** same object familiarization phase [Tukey post hoc (df, 18) p = 0.3700] and **(E)** novel object recognition phase [Tukey post hoc (df, 18) p = 0.1527]. **(F)** Novel object recognition of adult *female* offspring is independent of treatment [Tukey post hoc (df, 18) p = 0.0947]. n=7-11 animals per treatment.

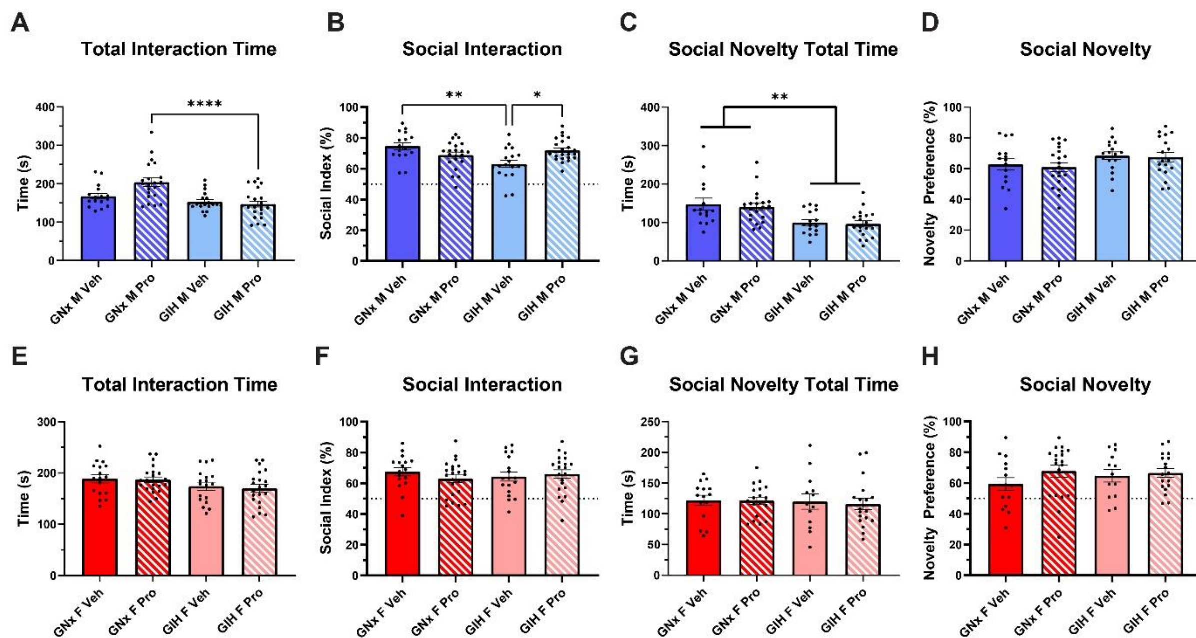

Figure 5: Propranolol rescues GIH-induced male-specific social approach deficits in adult male offspring. (A) Total interaction time during social interaction period of adult *male* offspring with both the social and empty chamber increased by prenatal propranolol exposure relative to GIH treatment groups [Tukey post hoc (df, 51) p<0.0001]. **(B)** Social interaction index of adult *male* offspring is significantly impaired by GIH exposure alone, but not with combined treatment [Tukey post hoc (df, 51) Propranolol p = 0.3069; GIH p = 0.0028; Combined p = 0.7927]. **(C)** Total interaction time of adult *male* offspring with both novel and familiar animals during social novelty period was significantly impaired by GIH and GIH combined treatment [Tukey post hoc (df, 43) Propranolol p = 0.6778; GIH p = 0.0037; Combined p = 0.0008]. **(D)** Social novelty preference of adult *male* offspring was independent of treatment [Tukey post hoc (df, 41) p = 0.8884]. **(E)** Total interaction time during social interaction period of adult *female* offspring with both the social and empty chambers was independent of treatment [Tukey post hoc (df, 51) p = 0.3327]. **(F)** Social interaction index of adult *female* offspring was unaffected by treatments [Tukey post hoc (df, 51) p = 0.5149]. **(G)** Total interaction time of adult *female* offspring with both novel and familiar animals during social novelty period was independent of treatment [Tukey post hoc (df, 41) p = 0.9906]. **(H)** Social novelty preference of adult *female* offspring was independent of treatment [Tukey post hoc (df, 41) p = 0.5154].

CHAPTER V

DISCUSSION

Summary

This dissertation builds on previous studies investigating the effects of intermittent hypoxia during gestation (GIH) on adult offspring cognition and behavior. The overall purpose of this work was to investigate the impact of GIH on offspring microglial in manners that may impair their ability to prune dendritic spines, leading to the previously observed behavioral phenotype in adult male offspring. In chapter 3, we assessed postnatal microglial number, phagocytic ability, and morphology and discovered a reduction in adult male offspring microglial complexity and ramification index. As the microglial ß2-adrenergic receptor has been implicated in blunted microglial surveillance and ramification, we expanded on this discovery in chapter 4 by targeting the receptor with an antagonist, propranolol, and assessed its influence on adult offspring cognition and behavior. We found propranolol rescued GIH-induced male-specific deficits in social approach behavior, but not their impaired long-term memory. The following discussion reflects on how these studies advance our understanding of GIH exposure and the overall influence of ß2-adrenergic signaling on microglial pruning capabilities.

Discussion

Throughout this thesis we have explored the role of GIH in offspring microglial development and proposed an interaction between the microglial ß2-adrenergic receptor and synaptic pruning. We addressed several hypothetical mechanisms behind the GIH-induced synaptic pruning deficits, and partially rescued the cognitive and behavioral deficits using a ß2-adrenergic receptor antagonist.

How GIH influences postnatal microglia

Our first hypothesis was that microglial number across postnatal development would be decreased in GIH offspring, leading to fewer microglia to prune excess synapses. The changes in microglial number observed across postnatal development had always been suspected, but never proven in rats. Previous work by our lab outlined the developmental trajectory in mice, and the rat timeline identified in chapter 2 follows the same pattern, but with different scales; mice experience an ~2-fold difference between P14 and other timepoints while rats are closer to 8-fold (Nikodemova et al., 2015). Importantly, microglial number is unaffected by GIH at any timepoint, and therefore our hypothesis that GIH offspring have fewer microglia was proven false (Figure 1). This finding has implications for our previously published inflammatory gene expression data as well, as the changes we have previously observed regarding increased inflammatory gene expression could potentially have resulted from a different number of GIH microglia relative to GNx rather than an increase in expression per microglia.

Our second hypothesis was that GIH offspring were impaired in their ability to phagocytose spines. The phagocytosis experiments in chapter 2 utilized fluorescent latex beads to determine microglial phagocytic capacity. As discussed in chapter 2, the precise mechanisms behind synaptic pruning are still being investigated, but we focused on complement opsonized beads as complement is one of the most well-established pathways of synaptic pruning. Despite performing several variations of phagocytic assays on GIH microglia, in no case did we detect a difference in GIH microglial phagocytic ability. Therefore, we concluded that our second hypothesis was also likely false, with the caveat that some microglia-synapse specific mechanism may be missed in our models (Figure 1).

More recent developments in phagocytosis flow cytometry experiments have developed pH-sensitive dyes such as pHrodo, which only fluoresce when exposed to an acidic environment such as the lysosome (Byun and Chung, 2018; Morini et al., 2021; Sellgren et al., 2019). This eliminates the confound of fluorescent beads or cell debris attached externally to the cell which we circumvent via trypan blue quenching of external beads. Using pHrodo, we could label neuronal dendrites in culture and then acutely expose them to microglia freshly isolated from both GNx and GIH offspring. Importantly, neuronal dendrites are typically cultured in serum-free media allowing us to avoid the complications of exposing microglia to serum discussed in chapter 3 (Ji et al., 2013). By exposing GIH microglia directly to neuronal dendrites rather than latex beads, we could eliminate the question of whether we are including a synapse-specific mechanism. Additionally, we could combine GNx neurons with GIH microglia, and vice versa to determine whether the deficits stem from a neuronal signaling molecule or a microglial receptor.

Our third hypothesis was that GIH microglia were less morphologically complex and therefore surveyed their environment less than their GNx counterparts. If microglia are unable to find and contact spines in need of removal, it will result in increased spine density in those brain regions. In chapter 3, we discovered GIH offspring microglia possess fewer branches, a lower ramification index, and decreased overall complexity (Figure 1). As ramification is directly related to overall surveillance, this supports my overarching hypothesis: GIH exposes fetal microglia to a bolus of norepinephrine during a critical period of development, resulting in long-term impairments to their surveillance and ability to contact and prune unnecessary dendritic spines.

Many studies have utilized colocalization of microglial Iba1 with post-synaptic marker PSD95 to measure the frequency of synaptic pruning events in fixed tissues (Comer et al., 2020; Osborne et al., 2021; Stevens et al., 2007; Wang et al., 2022). We wanted to take this experiment a step further by using this technique in adult GIH rats and were hoping to uncover a decrease in colocalization events. However, we ran into technical difficulties explained in the next paragraph that prevented us from completing these experiments optimally.

When performing any *in vivo* experiment, the goal is to use as few animals as possible, as wisely as possible. We had therefore decided to use the same brains previously fixed and sliced for dendritic spine analysis experiments for our Iba1-PSD95 colocalization experiments to conserve animals. We had previously detected an increase in spine density in these brains, so we hypothesized they would be ideal specimens for determining colocalization frequency and microglial morphology. These slices were 150µm thick to withstand the forced air injections of the gene gun when labeling dendrites for spine analysis. This thickness ended up being our downfall for the colocalization experiments, as the staining penetration and intensity of the synaptic marker varied wildly between slices. This prevented us from setting a consistent threshold when performing colocalization analysis using Imaris imaging software and prevented us from getting reliable results. On the bright side, an experimental design/methodology is now in place for future students to show a change in colocalization, and they know to use smaller slices within the 20-40µm range! Additionally, we were still able to utilize the Iba1 staining in the 150µm slices for morphological assessments, and from these results we obtained the decreased microglial complexity and ramification indexes presented in chapter 2. Overall,

I gained a wider understanding of everything that goes into imaging studies, the importance of pixel resolution in determining colocalization, and still got positive data through Iba1 morphology in the end.

The Neuronal Side of Dendritic Spine Density

It's important to note that while this thesis specifically explores the microglial side of dendritic spine maintenance, there is also the neuronal side driven by spine formation and autophagy. In fact, prior to the late 2000s, much of what we knew about synapse formation and elimination was completely independent of microglia (Schafer et al., 2012; Stevens et al., 2007; Wake et al., 2009). The role of neuronal autophagy in dendritic spine density is an entire research topic on its own and was the subject of Dr. Amanda Vanderplow's doctoral thesis in the laboratory of our collaborator, Dr. Michael Cahill. Briefly, dendritic spines can be eliminated through neuronal autophagy, which is modulated by neuronal mTOR signaling pathways (Tang et al., 2014). When mTOR is overstimulated, it prevents formation of the autophagosome early in the pathway resulting in decreased elimination through neuronal autophagy (Kim et al., 2011). Dr. Vanderplow identified an increase in mTOR signaling in adult GIH male offspring which she then inhibited using rapamycin, an mTOR inhibitor. In this manner, she rescued the increased spine density phenotype through a presumably neuronal mechanism via promoting neuronal autophagy (Vanderplow et al., 2022). Importantly, rapamycin treatment decreases dendritic spine density regardless of treatment type as while the rapamycin treatment rescued the GIH induced spine increases, it also decreased the GNx spine density levels sufficiently to induce cognitive and behavioral impairments in this treatment group (Vanderplow et al., 2022). Although the rapamycin treatment was able to rescue

GIH-induced cognitive deficits, it still may not be the entire picture. It is possible that GIH microglia have impaired pruning ability leading to increased dendritic spines, and the rapamycin treatment is simply compensating for the lack of pruning by increasing spine elimination rates through neuronal autophagy, an effect that is independent of microglial dysfunction. Hence, the research into GIH-induced microglial impairments still requires further investigation.

Targeting the ß2-Adrenergic Receptor with Propranolol

In chapter 4, we utilized the ß-adrenergic receptor antagonist propranolol concomitant with GIH exposure with the goal of blocking GIH-induced increases in norepinephrine from interacting with microglial ß2-adrenergic receptors. Additionally, we addressed the paucity in long-term studies of cognitive and behavioral outcomes in offspring exposed to prenatal propranolol. In doing so, we observed a significant increase in anxiety-like behaviors in offspring exposed to propranolol, suggesting a potential interaction with ß2-adrenergic receptors in mPFC excitatory neurons which develop during the latter half of gestation and play a key role in anxiety-like behaviors (Lei et al., 2022; Rice and Barone, 2000). This has implications for the ~3% of pregnancies treated with ß-blockers for hypertension and may help direct treatment towards the commonly used ß1-adrenergic receptor specific therapies rather than the non-selective ones like propranolol (Bergman et al., 2018; Duan et al., 2017). Additionally, we found prenatal propranolol treatment selectively rescues GIH male offspring social approach deficits but not long-term memory impairments (Figure 1).

Different behaviors are controlled by different regions of the brain. For example, spatial working memory is typically considered a prefrontal cortex driven process (van Asselen

et al., 2006). Socially driven behaviors are typically the realm of the prefrontal cortex and the amygdala, while long-term memory tests like the novel object recognition test is controlled through the prefrontal cortex and the hippocampus (Amaral, 2003; DeVito and Eichenbaum, 2010; Franklin et al., 2017; Hammond et al., 2004). It is important to note that while we observed an increase in dendritic spine density of the mPFC of adult male offspring, we did not see an increase in hippocampal spine density. As the mPFC is involved in all three of these behaviors, it is tempting to speculate that the increase in its spine density is the root behind all the problems. However, biology is rarely as simple as we want it to be. It is possible that the GIH-induced cognitive impairments for the social and long-term memory behaviors act through separate mechanisms, and the fact that we observe a selective rescue of the social but not long-term memory impairments suggests we have addressed only one of these.

While rapamycin was able to rescue GIH offspring cognitive impairments when given as a postnatal treatment, propranolol exposure prevented offspring social deficits when given concomitant with the original GIH stimulus. The fact that prenatal propranolol exposure alone does not improve social interaction but still protects against GIH social approach deficits suggests that propranolol selectively counteracts the GIH deficit in social approach behaviors by targeting the GIH mechanism of action rather than treating a symptom of the GIH deficit like their dendritic spine levels.

We also planned to explore the effects of prenatal propranolol with and without GIH on spine density in adult offspring, but we ran into technical hurdles explained in the following paragraph that prevented completion of the experiments. While we couldn’t complete the experiment, I learned an incredibly valuable lesson from the experience – always verify

something works correctly, and the results are as they should be, before doing it to all your samples.

The dendritic spine analysis project involved a lot of new techniques for me including how to label spines, how to image them, how to analyze the images using neuron studio software, etc. I was trained how to perform each task, performed said task on my samples, and continued onto the next task...without having anyone check the results of the previous. (Can you see where this is going?) After spending roughly 4 months staining, taking images, analyzing them, and finally sorting through the data, the GNx and GIH control results did not show the previously identified increase in dendritic spine density. I went through the whole staining and imaging process again with our collaborators Dr. Mike Cahill and Dr. Amanda Vanderplow step by step, and we discovered that the Watters lab user login for the microscope had different imaging settings set from the Cahill lab login that I had been trained on. This resulted in all the images I had taken being distorted. Not a large amount that was immediately obvious from any single image, but when viewed on the microscope it was immediately apparent to Amanda who had done hundreds of hours of spine imaging that something was off. If I had taken the time to confirm my images looked good after 3-4 images were taken and analyzed, rather than wait until all 200+ images were done, I could have saved myself months of trouble. I learned then and there to always validate the results in small batches before proceeding with the entire experiment. Cue me re-staining and re-imaging all the samples over the next two months. After re-imaging, we then learned about a critical flaw in the samples themselves – they had undergone behavior testing before fixation. It turns out that behavior testing, being a new experience for the rodents involving social interaction and long-term memory testing,

generates new dendritic spines (Michelsen et al., 2007). As our main endpoint is an increase in dendritic spines in GIH offspring, this presented a confound that prevented further analysis as the GNx offspring would theoretically experience a greater increase in spine density than the GIH who may reach a ceiling effect, extinguishing the difference in density between treatments. Again, this could have all been prevented by further communication between me and our collaborators during the experimental design stages. Overall, I learned a ton about imaging, imaging analysis, and dendritic spines from this project, but more importantly, I learned the value of thorough research and communication BEFORE starting a research project, as well as to verify, step by step, that a protocol is working before diving headlong into a full experiment in a methodology I've never done before. I then used this knowledge to an (almost annoying) degree when performing the Iba1 morphology and fractal analyses with the Ulland lab for the Chapter 3 morphological data by asking Kaitlyn to check, double check, and please just one more time confirm that my images would work for fractal analysis and that my outputs look correct. It ended up saving me some time there as we learned the microscope that I was originally going to take images on did not have a powerful enough resolution for colocalization and fractal analyses. A quick switch to the confocal over at WIMR and everything worked beautifully, resulting in the morphological data presented in chapter 3.

Future Directions

The experiments throughout this thesis propose a direct role for ß2-adrenergic receptor-norepinephrine signaling in microglial synaptic pruning. Complement 3 binds synapses in an activity-dependent manner to identify them for elimination (Schafer et al., 2012). Complement 3 concentrations in the cortex follow a circadian rhythm, peaking at the onset

of the sleep phase and reaching a nadir before the wake period(Choudhury et al., 2020). This correlates well with surveillance of microglia increasing during periods of sleep due to reduced norepinephrine-ß2 adrenergic receptor signaling (Liu et al., 2019; Stowell et al., 2019), and allowing them to sense, contact, and eliminate synapses bound to complement. Additionally, 8 hours of sleep deprivation, and therefore consistently increased norepinephrine signaling, significantly increases complement 3 protein levels in cortical tissue homogenates, again suggesting a reduction in pruning due to a lack of sleep (Bellesi et al., 2017). The logical next step would be to prove that microglial ß2-adrenergic receptor signaling and synaptic density are directly related. The simplest, most direct experiment to prove this is to conditionally knock down and overexpress the microglial ß2-adrenergic receptor during peak pruning periods in a mouse model and assess their synaptic density in early adulthood. By knocking down the microglial ß2-adrenergic receptor, we would free microglia from their normal restraints during wake periods, allowing for increased pruning and therefore reduced synaptic density. Overexpressing the receptor or inducing a constitutively active variant would be a little more difficult as we risk desensitization coping mechanisms, but still should result in decreased synaptic density compared to wild type controls.

As a complementary endpoint to spine density, we could utilize the previously identified increases in complement 3 protein expression that occurs at the onset of the sleep period (Bellesi et al., 2017; Choudhury et al., 2020). By knocking down the microglial ß2-adrenergic receptor, we would theoretically lose the blunted surveillance and pruning during wake periods. Therefore, the resulting build-up of complement 3 during the wake period would no longer occur. Similarly, the decrease observed after sleep would be

absent from a model with a constitutively active microglial ß2-adrenergic receptor. In an additional experiment independent from microglial ß2-adrenergic receptor modulation, it would be interesting to further validate the build-up of complement occurring during sleep periods by switching the day/night cycle of rats and exploring their complement levels at different times of day prior to, during, and after the day/night switch. We could also measure expression of various circadian genes like clock genes to determine how quickly the complement levels adjust relative to other circadian mechanisms.

After confirming that the ß2-adrenergic receptor is directly responsible for blunting the microglial pruning response, we could take the experiment a step further with the GIH model. In an ideal scenario, I would take GNx and GIH offspring and perform thin-skull, live, unanesthetized microscopy. By observing their responses to sleep and wake states, I would expect to see less ramified GIH microglia during the sleep state compared to their GNx counterparts. I would also like to take this experiment even further and explore the microglial sleep/wake response of a control rat exposed to intermittent hypoxia while under the microscope to determine if sleep apnea itself can reduce microglial surveillance without the sleep fragmentation aspect. This would have profound implications regarding sleep apnea in young children and teenagers who are going through that peak pruning period, as well as in preterm infants who are rapidly generating new spines and exhibit higher than normal rates of sleep apnea (Lorenzo et al., 2021). Finally, the above experiments would connect two distinct fields of research: sleep and microglial pruning, potentially starting new and exciting collaboration opportunities. Given that sleep patterns vary so wildly across development from childhood to adolescence to adulthood and aging

individuals, how microglia survey their environments and prune during these different developmental periods is of great importance.

The implications of this ß2-adrenergic receptor-pruning relationship extend not just to postnatal development, but also to diseases of neurodegeneration like Alzheimer's Disease (AD). In AD patients, the locus coeruleus is one of the first regions to experience degeneration, leading to a decrease in norepinephrine throughout the brain (Evans et al., 2020; González-Prieto et al., 2021). Interestingly, AD patients prescribed ß-blockers exhibit worsened symptoms and faster disease progression, and administration of ß-blockers to AD mouse models potentiate CNS inflammation (Evans et al., 2020). It is my hypothesis that this is due to blocking microglial ß2-adrenergic receptors, freeing them from their normal surveillance constraints and allowing them to more readily interact with the AD environment, leading to increased reactivity, inflammation, and pruning of synapses leading to potentiated synaptic loss. In addition to the effects of knocking down specifically microglial ß2-adrenergic receptors on normal developmental synaptic pruning, I propose that its knockdown in an AD mouse model would accelerate the neurodegenerative process like those observed when using ß-blockers, and that the degeneration is due to a microglia-specific interaction. Similarly, by using DREADDS to selectively overexpress or over activate microglial ß2-adrenergic receptors only during later stages of AD progression, I believe that we could reduce microglial neuroinflammation and synaptic loss.

Conclusions

Sleep disordered breathing during pregnancy is incredibly common and is found to be more and more prevalent among high-risk pregnancies like those of obese or

hypertensive women (Johns et al., 2020). Despite this, we know comparatively little about its long-term consequences. The goal of my research, and our lab, is to shed some light on the unknown aspects of sleep disordered breathing. The work comprising this dissertation advances our understanding of GIH-induced alterations to offspring microglia. We identified morphological changes in GIH male offspring microglia resulting in reduced complexity and ramification, revealing a potential mechanism behind the cognitive and behavioral impairments observed in adult GIH offspring (Chapter 3) (Figure 1). Additionally, we explored the impacts of prenatal exposure to propranolol on offspring cognitive and behavioral outcomes and identified its protective effects against GIH-induced social interaction deficits (Chapter 4). Collectively, this thesis suggests a direct role for the microglial ß2-adrenergic receptor in pruning of dendritic spines which has implications for both developmental and neurodegenerative processes. Finally, as with all research from our lab, it suggests a need for further awareness and treatment of sleep disordered breathing during pregnancy.

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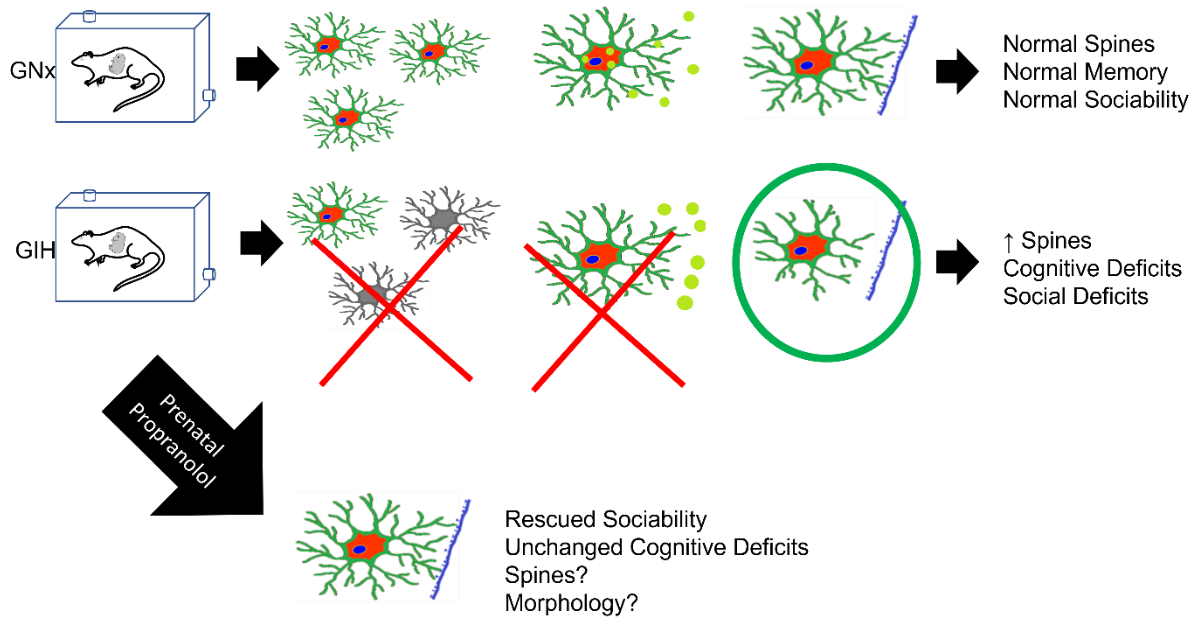

Figure 1: Pictorial representation of thesis results. GIH microglia exhibit normal number and phagocytic ability, but impaired surveillance. We hypothesize that propranolol concomitant with GIH prevents the morphological impairments leading to the observed rescued sociability in the offspring.

APPENDIX

Measuring Maternal Fecal Corticosterone after 3 Hours IH Exposure

METHODS

Fecal Corticosterone Measurements

Fecal corticosterone levels change in response to stress roughly 6-8 hours after they appear in serum, and collection of fecal pellets is a relatively non-stressful, non-invasive procedure compared to blood draws (Nemeth et al., 2016; Siswanto et al., 2008). Therefore, to measure corticosterone changes in response to GIH exposure, a separate cohort of dams were placed in fresh, clean cages when entering the gas system on GD10. All fecal pellets produced between 0-6 hours of exposure time were collected and processed for fecal corticosterone levels as a baseline pre-IH measurement. Fecal pellets produced between 7-9 hours after starting IH exposure were collected and processed to determine the initial stress response to hypoxia vs normoxia exposure. Fecal samples were stored at -20°C overnight and then dried at 50°C in an oven until weight no longer changed over a 15minute period. Next, 0.2mg of dried, powdered feces were then resuspended in 2mL 200proof Ethanol (Decon Laboratories) and gently shaken for at least 30minutes. Suspensions were then pelleted at 5000rpm/15min and 1mL of supernatant was removed and stored at -20°C. Supernatants were then evaporated completely using a speedvac to complete isolation. Samples were then processed per ELISA instructions using a corticosterone ELISA kit (#501320, Cayman Chemical Company). N = 10-14 dams per treatment.

RESULTS

GIH Induces a Trending Increase in Maternal Corticosterone Levels

Increased maternal stress during pregnancy is known to cause numerous long-term consequences for the offspring regarding cognitive and behavioral endpoints. To determine whether GIH exposure is inherently more stressful for the pregnant dams than GNx exposure, we measured maternal corticosterone levels prior to and just after the onset of gas exposure. Because blood draws are inherently stressful, and prenatal stress is something we wish to avoid, we chose to implement a method of corticosterone measurement that is as non-invasive as possible. Increased corticosterone levels have been identified in fecal pellets peaking roughly 8 hours post-stressor in rats (Nemeth et al., 2016; Siswanto et al., 2008). Therefore, we collected fecal pellets produced from 0-6 hours on the gas exposure system to use as a baseline and compared them to fecal corticosterone levels of those pellets produced from 7-9 hours post-exposure to measure early stress response to IH in pregnant dams (Figure 1). Data is presented as a percent change of the baseline corticosterone value from the same animal. While not significantly different, GIH offspring trend towards having an increased level of corticosterone (t (df, 22) = 1.707, P=0.102). It should also be noted that we collected additional pellets from 9-12hours post-exposure and saw no significant or trending differences between treatments suggesting that any increase in maternal stress levels is transient (data not shown).

DISCUSSION

As GIH has previously been associated with cognitive and behavioral deficits in adult male offspring similar to those observed in prenatal stress models, we hypothesized that GIH exposure may increase stress hormone levels in the dams (Vanderplow et al., 2022). We have identified a trending increase in GIH dam corticosterone levels during the first three hours of exposure, however this increase is ameliorated by 6 hours of exposure.

Whether this increase occurs consistently at the onset of each day of intermittent hypoxia throughout pregnancy or only on the first day is a matter for further study. Additionally, while the measured corticosterone increases are only trending, the corticosterone changes measurable in fecal pellets are less sensitive than when utilizing serum. Therefore, it is possible our trending differences in fecal stress levels may become significant if performed in serum samples instead. The trade-off being increased stress induced in the dams through the stress-measuring process.

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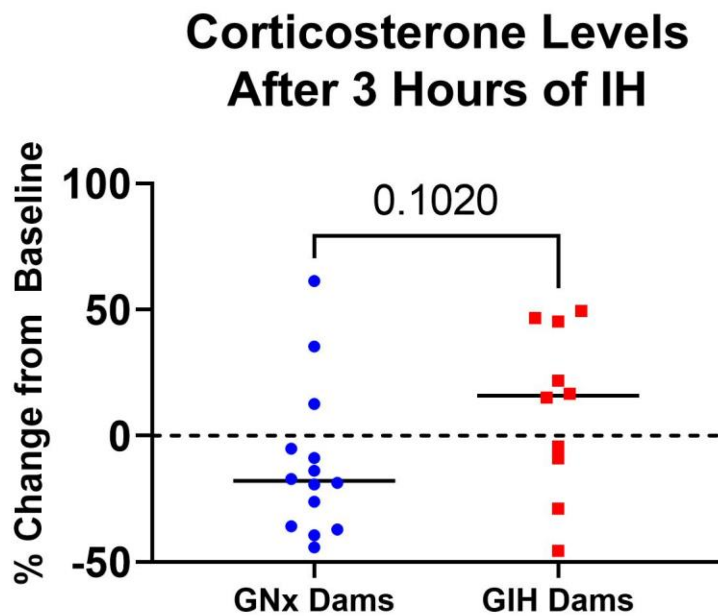

Figure 1: Three hours of intermittent hypoxia induces a trending increase in fecal corticosterone levels of pregnant rats. Data shown as a percent change from baseline after 3 hours of IH treatment [t (df, 22) = 1.707, p = 0.1020].