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

By

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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

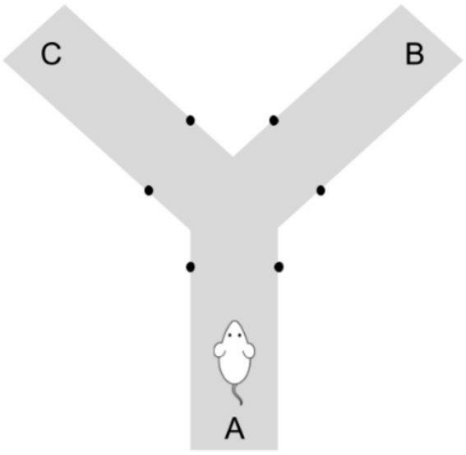

Figure 1: Y-maze Test depiction with rat starting in arm A.

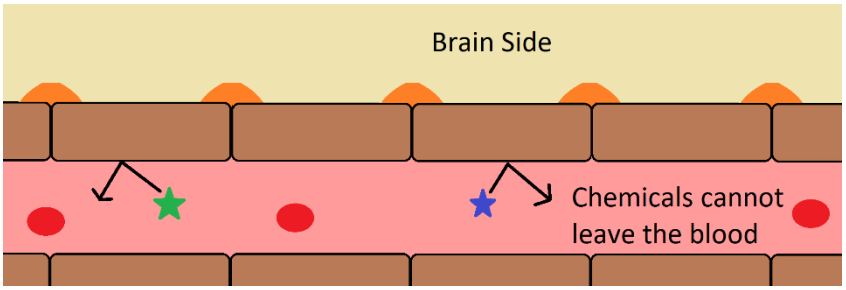

Figure 2 - Depiction of the blood-brain-barrier preventing chemicals from exiting blood and entering the brain

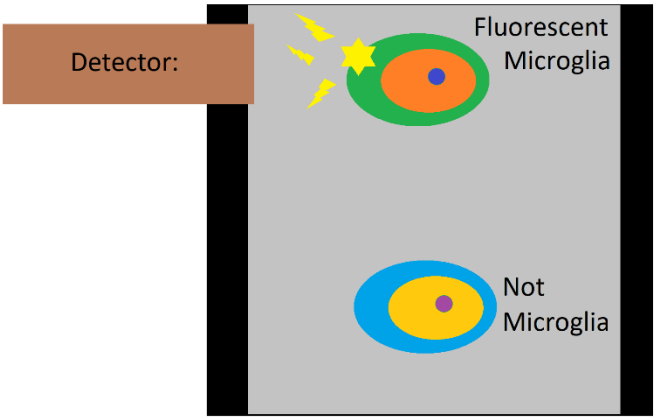

Figure 3: Depiction of a microglia and non-microglia cell going through the tube of a flow cytometer.

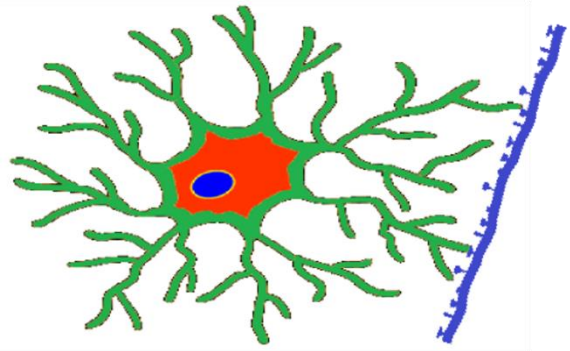

Figure 4: Depiction of a Ramified Microglia interacting with Synapses