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Maternal Infection can Affect Offspring Throughout Their Life, But Timing Plays a Role as to How

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ABSTRACT

Early brain development marks a period of vulnerability during gestation that when disturbed, can lead to changes in physical, mental, social and cognitive development in offspring, including risk for neuropsychiatric conditions such as schizophrenia or autism spectrum disorder (ASD) (Meyer et al., 2006). These disturbances have an incredibly strong link to maternal infection, but more specifically, the body's cytokine-associated immune response. Studies show a direct link between an excess of cytokines and changes (behavior reminiscent of ASD, schizophrenia, and obsessiveness) in the offspring, although cytokines aren't the only major factor at play. Different trimesters of gestation open up different periods of vulnerability to the developing fetus during which cytokines in the fetal brain may respond in different ways that can lead to dysfunction in the offspring (Meyer et al., 2006). One group in particular conducted a study of mice to demonstrate how infection during the first or second trimester of pregnancy can have different effects on the offspring and their behavioral development later in life.

Introduction

Maternal infection has been linked to an array of changes in the offspring ranging from physical to behavioral (Meyer et al., 2006). Infection however, is not always the direct cause of these changes, but rather the maternal immune response (Meyer et al., 2006). Few viruses are actually able to cross the placental barrier and affect the developing fetus, Zika is one of the exceptions which is why it poses such a risk to pregnant mothers (Rasmussen & Jamieson, 2020). In response to maternal infection, the body produces an excess of cytokines, proteins involved in the formation of the central nervous system (CNS), cell differentiation, expression of transmitters and brain development (Meyer et al., 2006). Unlike many viruses, cytokines have been found to cross the placental barrier and enter fetal circulation (Boksa, 2010) . The specific cytokines that are typically measured are IL-1β IL-6, IL-10 , and TNF-α, all of which are responsible for immunoregulation and mediate inflammation.

While it is unknown how exactly the cytokines are able to alter fetal development, studies show a direct correlation between spikes in cytokine production and alterations in the offspring (Vasistha et al., 2020). One study in mice has shown that when a pregnant dam is injected with cytokine-inducing treatments, the offspring are affected even though no maternal infection was actually present (Vasistha et al., 2020). Schizophrenia and autism spectrum disorder (ASD) are two prime examples that can arise as a result of maternal infection along with changes in social and cognitive behavior. The Developmental Origin of Health and Disease (DOHaD) theory supports the notion that the cytokine spike during prenatal development may even affect later life development, susceptibility to neuropsychiatric and cognitive disorders such as Alzhiemer's and Parkinson's disease, and stress reactivity (Bilbo & Schwarz, 2009). While this may be true, another study shows strong evidence that the timing of the infection is what differentiates symptoms in the offspring (Meyer et al., 2006). In other words, infection during the first trimester of pregnancy causes different effects in the offspring than an infection during the second trimester. For example, infection during the first trimester has been linked to suppressed spatial exploration, whereas infection during the second trimester

leads to more preservative and obsessive behavior (Meyer et al., 2006). This suggests that the development of the fetal nervous system has differing periods of vulnerability to maternal infection, which explains why the cytokine-induced response has significant effects on the offspring during the early to mid trimesters of human pregnancy.

A study conducted in 2006 sought to determine how the timing of maternal infection affects behavior in the offspring in a rodent model (Meyer et al., 2006). Pregnant mice were injected with polyriboinosinic- polyribocytidilic acid, also called PolyI:C, a synthetic protein that models double stranded viral RNA. The study split the pregnant mice into four groups, one that would be injected with the PolyI:C at gestation day 9, and the other that would be injected at gestation day 17, one group that would be injected with saline on day 9 or day 17, and a group that received no injections. The days of injection correlated to the first and second trimesters of human pregnancy to get an accurate representation of how human offspring would react to the differing infection days.

The study hypothesized that because the maternal immune system naturally fluctuates during pregnancy, cytokine responses to maternal infection could alter the developing fetal immune response and allow different types of cytokine in the fetus to alter brain and CNS development, thus leading to neurocognitive changes in the offspring (Meyer et al., 2006). Addressing this hypothesis is crucial to understanding the development of offspring and the relationship between maternal infection and fetal cytokine production. This connection may shed light onto how exactly cytokines can cause changes on a cellular level and influence fetal development.

Materials and Methods

Mice included in the study were bred two weeks after being introduced to their new housing then injected once at the intravenous route at the tail vein with either a vehicle solution, Poly:IC or neither. Mice were injected on either gestation day 9 or gestation day 17 to mimic the first and second trimesters in human pregnancy. To then assess how cytokine levels in maternal serum and fetal brain tissue, pregnant dams were then killed 6 or 3 hours after receiving an injection. Trunk blood was then collected and fetal brains were removed for a cytokine protein assay. Total RNA was isolated from fetal brain samples. Gene expression assays were performed for IL-1β IL-6, IL-10 , TNF-α. Cytokine levels were measured in the maternal serum and fetal brain samples multiplexed particle-based flow cytometric cytokine assay. Once the offspring were 24 days old, eight Poly:IC exposed and four controls (saline exposed) were sacrificed for immunostaining. Horse anti-doublecortin, rabbit anti-cleaved caspase-3, rabbit anti-GFAP, and mouse anti-Reelin antibodies were used to detect the proteins of interest in the mice: IL-1β IL-6, IL-10 , TNF-α. Fifteen coronal brain samples were taken from each animal, along with the immunostained samples. From the samples, the total number of Reelin-positive neurons in the CA1 stratum orien region could be determined. The neurons in this region are involved in fear conditioning and fear learning (Meyer et al., 2006). Reelin-positive neurons in the dentate gyrus, a part of the brain where senses merge together and play a crucial role in memory and learning, were also measured. In the developing fetal brain, and also in the mature adult brain, Reelin plays a role regulating neuronal migration and dendrite growth and branching (Jossin, 2020). Low levels of Reelin in the samples could potentially mean an underdeveloped brain, which would cause numerous neuropsychiatric problems in the offspring, as is also seen with humans in postmortem studies (Meyer et al., 2006). Caspase-3 positive cells were also measured in the dentate gyrus. Activated Caspase-3 plays a central role in completing apoptosis (Porter & Jänicke, 1999).

At 24 days, pups were weaned and separated by sex for a series of behavioral assays that took place when they reached 14-16 weeks old. The first test was an open field exploration test with four 40 x 40 cm^2 white arenas. Offspring were placed in the center (measured 13.5 x 13.5 cm^2) and allowed to explore the arena for 1 hour (both sexes were in the same arena). The second behavioral assay focused on discrimination reversal learning. The experiment used a white circular tank with a diameter of 1 meter, and filled 19 cm with water. Opaque plexiglass dividers were placed in water in the shape of a ‘T’, reaching 18 cm above the surface. The start of the T was 30 cm long while the parallel choice arms were 45 cm. At either the end of the right or left goal arm was a cylindrical piece of plexiglass with a diameter of 7 cm and a height of 18.5 cm, which served as the escape platform if the mouse had chosen the ‘correct’ arm. For the series of trials, mice were taught to differentiate between the right and left arms of the T. Half

of the mice in each group had the escape platform on the left, while the other half had it on the right. Six trials were conducted daily, with 10 minutes between each one. The mice had up to one minute to decide between going to the right or left arm and once making the choice, were physically blocked from trying to go to another arm. If the mouse made it to the escape platform, it was allowed to be there for 10 seconds, if it chose the wrong route, it had to stay confined in the arm for 10 seconds. The next part of the experiment took place once mice had successfully chosen the correct arm at least 11 times across two days. The escape platform was then switched to the opposite arm to see reversal learning in the mice. The training continued until each mouse had successfully chosen the correct arm at least 11 times. It should be noted that only male mice were used for this experiment in particular, since females had excessive anxiety, or floating behavior.

To analyze the data collected from the tests, the study used parametric ANOVA, and Fisher's LSD *post hoc* comparisons whenever there were any statistically significant finds. Both ANOVA and Fisher's LSD ensure that there are no prominent outliers in the data, and allow for the comparison of multiple groups, specifically between the placebo and dose group (Meier, 2006). Offspring who received the vehicle dose were also grouped in with offspring who received no dose since the results did not differ. For immunostaining, the sex of the mice was not included because the results were consistent with both sexes.

Results

The results of the behavioral exploration test showed that offspring exposed to Poly:IC on day 9 had decreased exploration compared to the control group and the offspring exposed on day 17. Offspring on day 17 exhibited preservative behavior, but not decreased spatial exploration. It should be noted that offspring exposed on day 7, did not exhibit preservative behavior. Spatial exploration was measured by frequency to enter the center part of the field. The specific effect on GD9 mice cannot be attributed to locomotor changes since the total distance traveled by all of the groups never differed. Instead, the authors suggest that this represents immune activation in response to the maternal infection. In the discrimination reversal learning task, all groups were able to discriminate between right and left without difficulty. This is seen by the percentage the mice got correct on training days. GD17 had a clear selective deficit when the correct platforms were switched, which is evident of a preservative phenotype. All groups had the reversal effect, however it persisted to the second day in solely the GD17 mice. Difficulty during the reversal phase, but not the initial phase, is indicative of preservative behavior, seen in schizophrenia, ASD, obsessive compulsive disorders, and addictive behavior (Meyer et al., 2006). There was no difference seen between any of the groups during the initial phase, which suggests that the difficulty experienced by GD17 mice was not a learning deficit. Similar behavior was also observed in the GD17 mice during their daily performances, especially when it came to choice accuracy.

Brain samples and immunostainings from the groups confirmed the hypothesis that maternal infection can lead to neurocognitive changes. The samples revealed that offspring exposed to Poly:IC and killed 24 days after birth, had lower Reelin expression in the hippocampus than the control group. The effect was most pronounced in offspring exposed to Poly:IC at GD9, where expression was particularly low in the stratum oriens of the dorsal CA1 subfield. On the other hand, offspring that were exposed on GD17, did not have as significant a lack of expression. Since Reelin is also involved in fetal and postnatal brain development, the lack of expression across the brain samples suggests there might also be other changes in expression of neuronal progenitor cells. Neuronal progenitor cells (NPCs) are crucial to the development of the central nervous system as they give rise to almost all of the glial and neuronal cells that are found in the CNS (Meyer et al., 2006). Lack of these cells would suggest that the offspring have underdeveloped central nervous systems. This finding was confirmed from the immunostaining samples, which, using an antibody against DCX to view them, showed reduced NPCs in the dorsal genate gyrus, regardless of the timing the sample was taken. Both offspring exposed on GD9 and GD17 had significantly lower levels of NPCs than the control group. After finding low levels of Reelin, the authors hypothesized that they would then find higher levels of cell loss along with higher levels of Caspase-3. However, increased apoptotic activity was only found in offspring exposed on GD17.

Apoptotic activity in GD9 was somewhat reduced compared to the control group, although the numbers are not significant. Despite the changes in levels of Reelin and Caspase-3, the authors explain that in line with previous studies, the offspring exposed to Poly:IC did not have any morphological changes in their hippocampus or excessive cell death.

With regards to cytokine levels, the study found that all four of the cytokines tested –IL-1β IL-6, IL-10 , TNF-α – significantly increased in the maternal serum after the Poly:IC injection. The effects of the Poly:IC were more pronounced in the samples taken three hours after the injection compared to the ones taken six hours after. The maternal response to IL-10 and TNF-α at three hours was stronger in the GD9 mice rather than the GD17. However, the response to IL-6 and IL-10 was greater in GD17 after six hours, compared to the response in GD9 after six hours. Animals that were injected with the vehicle solution never had detectable amounts of IL-1β, regardless of the timing of injection. Mice also never had any detectable amounts of IL-6 when they were injected with the vehicle solution on GD17, whereas they had very low levels, but detectable levels, if they were injected with the vehicle solution on GD9.

Next, the levels of IL-1β IL-6, IL-10, and TNF-α were tested in the fetal brain samples taken three or six hours after an injection of Poly:IC or vehicle solution. The middle line on the graphs represent levels of the four cytokines in the control and vehicle groups, any amount that increases or decreases compared to the mean, is shown as a bar graph. At three hours after the injection on GD9, IL-10 levels in the fetal brains actually decreased, then were again seen as matching the control line in samples taken six hours after injection. GD9 samples also saw a drastic increase in IL-6 in samples taken three hours after injection, however were at much lower levels in the six hour samples. GD17 samples also saw a rise in IL-1β during the first three hours, but an opposite in the GD9 samples, which actually had decreased levels of IL-1β. However, in the six hour samples, IL-1β had increased for GD9 in comparison to GD17 and the controls. GD17, on the other hand, had levels lowered back to the control line. For the TNF-α, no statistically significant differences were observed for either GD9 or GD17 in the three-hour samples. Both GD9 and GD17 samples taken after six hours saw an increase in TNF-α, the higher levels in GD17.

The authors conclude that mice exposed to Poly:IC on GD9 show decreased spatial exploration, while the mice exposed on GD17 exhibit preservative, repetitive and continuous, behavior (Meyer et al., 2006). However, each deficit was not seen in the other group. The results also suggest that the prenatal immune activation in response to maternal inflammation can lead to specific neurocognitive traits, but that the specificity depends on the timing of the immune activation. These findings prove the study's hypothesis that maternal inflammation can indeed affect the offspring's neurocognitive development. The results also support the notion that fetal development has differing periods of vulnerability, thus leading to different deficits depending on the timing of infection. The study also notes that the reduction of hippocampal Reelin expression was most notable in mice exposed on GD9, while mice exposed on GD17 had an increase in apoptosis. Both of which are seen in the disease process of ASD and schizophrenia (Bilbo & Schwarz, 2009). The cytokine-related inflammatory response was a potential cause of these specific outcomes, which explains why timing was so important. The authors also specify that no actual neurodegeneration was observed in the fetal brain samples, which further points that the effects seen were from neurodevelopmental disturbance, something central to the process of schizophrenia and autism (Meyer et al., 2006). The study also states that it provides the first evidence that maternal inflammation at different gestation periods produces unwanted cytokine responses in the fetal brain shortly after exposure.

Discussion

Overall, the study design addressed the hypothesis and was very thorough in looking for an answer to how timing plays a role in the specific outcomes of offspring exposed to maternal inflammation and cytokines. The authors look at multiple samples for cytokine presence, including maternal serum and fetal brain tissue, as well as analyze the behavior of the mice. If the study was more specific to cytokines and not the effects of timing of infection, also testing

for the presence of IL-1β IL-6, IL-10 , and TNF-α in the placenta of pregnant mice would give insight into the possibility of interaction between maternal cytokines and the fetal immune system. The placenta could have been sampled three or six hours after the Poly:IC or vehicle injection, or at birth in the groups of mice that were not sampled early on. While the study shows evidence of increased cytokines in fetal brain tissue, it does not state if those are of fetal or maternal origin. In other words, were the cytokines produced by the fetal immune system in response to maternal infection, or did they cross the placental barrier and come into fetal circulation from the maternal serum? The study shows a noticeable change in behavior in offspring exposed to Poly:IC, especially during the water T-maze. DOHaD theory, mentioned earlier, also has something to add in terms of behavior. DOHaD theory claims that early-life exposure to cytokines can affect later-life susceptibility to neurodegenerative diseases such as Alzhiemer's or Parkingson's disease (Bilbo & Schwarz, 2009). While the study specifies there was no neurodegeneration present in the fetal brain tissue, there is still the possibility of it occurring later in the offspring's life. Alzhiemer's has characteristic cognitive deficits caused by tangles and amyloid plaques in the brain which affect the patient's spatial awareness, learning, judgment, and other executive functions (KJ et al., 2009). In mice, there are specific behavioral tests that can search for these deficits, such as spatial memory tests like the Morris or Radial Arm Water Maze, recognition memory tests like Novel Object Recognition, and associative learning tasks such as passive avoidance (KJ et al., 2009). The Morris Water Maze works by placing a rodent in cloudy water in order to incentivize them to swim to a hidden platform and escape. Cues are given as to the location of the platform, which tests the animal's ability to recognize and remember necessary information. Object recognition tests a rodent's memory by placing them in an environment with new objects, then showing them objects one by one, including some new objects, and observing the response to see if the rodent shows signs of recognition towards the repeated objects. Finally, associative learning tasks often involve either forcefully or passively teaching an animal to avoid a certain environment or object, then reintroducing them to the same environment or object and observing how fast they can avoid it. After the behavioral tests have been completed, the study should take samples of the entorhinal cortex and hippocampus to find amyloid plaques. The presence of amyloid plaques and tangles, along with results from behavioral tests, will provide evidence of the development of Alzhiemer's disease in the mice. If the offspring from the group GD9 or GD17 have a significant amount of amyloid plaques and exhibit dementia behavior in comparison to the control and vehicle group, it will be sufficient proof that maternal infection can affect the neurodevelopment of the offspring all the way to late life. Other than leaving out late life changes, the study did well in analyzing the earlier changes caused by exposure to Poly:IC in mice. However, one question that remains undiscussed is whether all of the Poly:IC exposed mice experienced the same symptoms and if their brain samples all showed the same results. The authors claim that overall the study showed these results, but they do not mention if there are any offspring that are born without these deficits. While it's possible that all offspring had some effect, the study leaves out the possibility that some were unaffected.

The study is ultimately successful in showing that maternal infection can lead to neurocognitive changes during fetal development and can even affect offspring during their early to middle life. The results of this study can be applied to human pregnancy, maternal infection, and fetal neurocognitive development. This shows great importance in understanding how the central nervous system and brain are formed with varying windows of vulnerability, and can also lead to having an overall better understanding of fetal development. Symptoms shown by the mice are a good segway into research on ASD, schizophrenia, and other mood disorders whose origins are suspected to be neurocognitive and can arise from altered brain development. The information gathered on the presence of cytokines in maternal serum and in fetal brain tissue can potentially help scientists to understand how cytokines interact with maternal infection and what specific reactions they can cause in the brain. Since it is still unknown how cytokine interaction with the fetal CNS and brain can cause disruption during development, the results from the study can shed light on the effects of these interactions and also the levels of cytokines present. Future studies on the subject of the timing of maternal infection could recruit pregnant human mothers who have unfortunately been diagnosed with the common flu sometime over the course of their pregnancy. Samples of maternal serum, cord blood, and placenta could be taken in order to measure the levels of IL-1β IL-6, IL-10, and TNF-α present. This would help explain how the body reacts to infection and how it alters cytokine production in humans. It would, however, be necessary to test the maternal

serum of each participant the same number of days after the infection so the study remains consistent. In regard to the children, analyzing both early and adolescent years is important to see if they develop any neurocognitive changes due to their early exposure to cytokines. These changes would include what was seen in the mice such as decreased spatial exploration and obsessive-type behavior.

The study set out to answer the question of how differently timed exposure to the maternal immune response can affect mice offspring in both levels of expression in their brain tissue and in behavioral changes. Ultimately, it was concluded that mice exposed to Poly:IC on GD9 experienced decreased spatial exploration, whereas mice exposed on GD17 had preservative behavior. Neither group, however, had the other group's deficits. Cytokines were also found in the fetal brain tissue and maternal serum of Poly:IC injected mice, showing a major difference between the injected mice and the control group. The study should have looked at the late life effects seen in the mice to branch off of DOHAD theory, instead of ending the study when the mice were mature. Analyzing late life behavior and brain tissue for symptoms of Alzhiemers and dementia in both groups could show how early exposure to cytokines have an effect even towards the end of life. Future research should turn towards analyzing prenatal human exposure to cytokines in an effort to further understand how exposure can shape behavior and alter neurocognitive development in children and adolescents.

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