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Prenatal stress affects placental cytokines and neurotrophins, commensal microbes, and anxiety-like behavior in adult female offspring

Tamar L. Gur [a,b,c,d,*], Lena Shay [a], Aditi Vadodkar Palkar [a,d], Sydney Fisher [a,d,e], Vanessa A. Varaljay [e,f,g], Scot Dowd [h], Michael T. Bailey [d,e,f,g]

[a] Department of Psychiatry & Behavioral Health, Wexner Medical Center at The Ohio State University, United States
[b] Department of Neuroscience, Wexner Medical Center at The Ohio State University, United States
[c] Department of Obstetrics & Gynecology, Wexner Medical Center at The Ohio State University, United States
[d] Institute for Behavioral Medicine Research, Wexner Medical Center at The Ohio State University, United States
[e] Center for Microbial Pathogenesis, The Research Institute, Nationwide Children's Hospital, The Ohio State University, United States
[f] Biosciences Division, College of Dentistry, The Ohio State University, United States
[g] Department of Pediatrics, Wexner Medical Center at The Ohio State University, United States
[h] Research and Testing Laboratory and Medical Biofilm Research Institute, Lubbock, TX 79407, United States

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ABSTRACT

Recent studies demonstrate that exposure to stress changes the composition of the intestinal microbiota, which is associated with development of stress-induced changes to social behavior, anxiety, and depression. Stress during pregnancy has also been related to the emergence of these disorders; whether commensal microbes are part of a maternal intrauterine environment during prenatal stress is not known. Here, we demonstrate that microbiome changes are manifested in the mother, and also found in female offspring in adulthood, with a correlation between stressed mothers and female offspring. Alterations in the microbiome have been shown to alter immune responses, thus we examined cytokines *in utero*. IL-1β was increased in placenta and fetal brain from offspring exposed to the prenatal stressor. Because IL-1β has been shown to prevent induction of brain derived neurotrophic factor (BDNF), we examined BDNF and found a reduction in female placenta and adult amygdala, suggesting *in utero* impact on neurodevelopment extending into adulthood. Furthermore, gastrointestinal microbial communities were different in adult females born from stressed vs. non-stressed pregnancies. Adult female offspring also demonstrated increased anxiety-like behavior and alterations in cognition, suggesting a critical window where stress is able to influence the microbiome and the intrauterine environment in a deleterious manner with lasting behavioral consequences. The microbiome may be a key link between the intrauterine environment and adult behavioral changes.

1. Introduction

Adverse prenatal events, including maternal stress, have the capability of negatively influencing the neurodevelopment of the fetus, with long term behavioral implications (Bale et al., 2010). The interplay between intrauterine growth factors, hormones, and the immune system is dynamic and integral to healthy development in the offspring. The microbiome is a potential contributor to this interaction. Stress is known to alter the inflammatory state of tissues, such as the spleen (Bailey et al., 2009; Avitsur et al., 2002, 2005; Devoino et al., 2004) and to promote the release of inflammatory mediators and cytokines from these tissues (Avitsur et al., 2005, 2002). In previous studies, changes in the gut microbiome have been associated with increases in inflammatory cytokines (Campos et al., 2016; Souza et al., 2004; Bailey et al., 2011; Gur and Bailey, 2016). However whether stressor-exposure increases placental inflammation, and whether this is associated with dysbiosis, has not been shown. While it is becoming increasingly clear that stress can alter the microbiome (Bailey et al., 2011, 2010), and the microbiome has the capability to influence behavior (Lyte et al., 1998; Cryan and Dinan, 2012; Kelly et al., 2016), direct

* Corresponding author at: IBMR Building, 460 Medical Center Drive, Room 253, Columbus, OH 43210, United States.
E-mail address: tamar.gur@osumc.edu (T.L. Gur).

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evidence for prenatal stress altering intrauterine microbes, inflammation, and neurodevelopment has been elusive.

Recent studies have reported that the placenta harbors a unique microbial population, though it is low in abundance (Aagaard et al., 2014). Exposure to stress alters the composition of the intestinal microbiome, with increased translocation of microbes from the GI tract to internal organs (Bailey et al., 2011, 2006; Bailey and Coe, 1999). If stressor exposure also increases translocation of microbes to the placenta and subsequent dysbiosis, perhaps infants exposed to antenatal stress are exposed to increased and altered populations of microbes *in utero*. Since there is mounting evidence that gut microbes can affect brain biology and behavioral responses, including anxiety-like, depressive-like, and social behavior (Cryan and Dinan, 2012), dysbiosis during gestation may influence neurodevelopment in a way that renders the individual susceptible to psychopathology. However whether stressor-exposure increases placental inflammation, and whether this is associated with dysbiosis, has not been shown. Indeed, disorders such as depression, schizophrenia, anxiety, and autism have been found to be associated with *in utero* and early neonatal exposure to these stimuli (Bale et al., 2010). The majority of studies to date have investigated the role of the hypothalamic-pituitaryadrenal (HPA) axis and epigenetic alterations as major contributors to psychopathology, and now the microbiome is an emerging candidate as a potential mediator of stress-induced pathogenesis.

Here we present the highly novel, and integrative findings that maternal stressor exposure alters maternal microbiota, influences inflammation and neurodevelopment *in utero*, with long lasting impact on behavior and microbiome in adulthood. Alterations in microbes bequeathed from mother to offspring therefore may explain an element of the transmission of maternal mental illness to the next generation. The effect of prenatal stress on offspring is sex-dependent in terms of epigenetic, immune system, and hypothalamic-pituitaryadrenal (HPA) axis regulation (Bale et al., 2010; Bale, 2011, 2009). BDNF has also been shown to have influences on behavior that are sex-dependent (Monteggia et al., 2007), and stress impacts BDNF differently in males and females (Franklin and Perrot-Sinal, 2006). In the current study, gestational stress led to different behavioral effects in males and females, with only the females displaying anxiety-like behavior. Because we chose to focus our manuscript on the development of anxiety-like behavior, and because anxiety is more common in women (Kessler et al., 2005), the present study focused on the response of females to prenatal stress. Data from male offspring will be included in future studies.

2. Methods

2.1. Animals

Female C57/Bl6 mice obtained from Jackson Laboratories were bred at Ohio State University Wexner Medical Center. Pregnant females were randomly assigned to either the stressed experimental group or non-stressed control group. The stressed group underwent restraint stress between embryonic days (E) 10-E16 for a period of 2 h between the hours of 09:00 and 12:00, using an acute restraint stressor. The restraint stressor involves placing a mouse in a 50 mL conical tube with perforations to allow for ventilation. At the end of the 2-h period, mice were removed from the device and left undisturbed until the following day when stress was repeated. A cohort of pregnant females was sacrificed at E17.5 for tissue collection; a different cohort of pregnant females was allowed to continue through parturition for behavioral testing, microbiome sampling, and tissue collection in offspring. Animals were weaned at 21 days and separated into female and male cages at that time. Behavioral testing was undertaken between P60-P70. Only female offspring were included in the present report. Male offspring were included in a separate study, as they demonstrated different behavioral, microbial, immunological, and neurobiological changes in response to prenatal stress. All experiments were conducted in accordance with the principles and procedures outlined in National Institutes of Health Guidelines for the Care and Use of Experimental Animals and were approved by the Institutional Animal Care and Use Committee at The Ohio State University.

2.2. Behavioral analysis

2.2.1. Elevated plus maze

The elevated plus maze (EPM) was used to assess anxiety-like behaviors. The apparatus was elevated 75 cm above the floor and exposed to fluorescent lighting. At the beginning of each trial, a mouse was placed onto the central area of the maze, facing an open arm. Mice were tested for a period of 5 min and their movements on the maze were tracked and subsequently analyzed by the Noldus EthoVision video tracking system. The following parameters were assessed: time spent in open and closed arms and distance travelled in open and closed arms. For the offspring, a sample of 14 non-stressed and 12 stressed animals from 5 separate dams were examined. For behavioral testing in the dams, 7 stressed and 7 non-stressed dams were examined on day E17.

2.2.2. Novel object recognition

The Novel Object Recognition (NOR) test is used to evaluate the ability to recognize a novel object in an environment as well as a component of neophobia (Antunes and Biala, 2012). The NOR test consists of two phases: familiarization and test phase. During the familiarization phase, two identical objects (in this case, two orange Pyrex bottle caps) are placed at opposite ends of an otherwise empty cage containing clean bedding. Mice are allowed to explore the cage and the two identical objects for 10 min. Mice are placed in their home cage at this time. Three hours after this phase, a novel object (small cell culture flask) is placed in the cage, in the place of one of the original objects. The mouse is placed in the center of the cage and allowed to explore for 5 min. Novel object preference is assessed using time spent investigating original object and the time spent investigating novel object, in relation to the total time spent investigating both objects (as opposed to the time spent in other areas of the cage). Cages and bedding were replaced after each trial. A sample size of 6 mice per group from 3 different dams was used.

2.2.3. Tail suspension test

Mice were individually suspended by the tail to a horizontal ring-stand bar (distance from floor, 35 cm) using adhesive tape affixed 2 cm from the tip of the tail. Mice demonstrated several escape-oriented behaviors interspersed with bouts of immobility as the session progressed. A 5 min test session was videotaped and scored by a trained observer who was blind to the experimental conditions. The behavioral measure scored was the duration of "immobility," defined as the time when mice were judged to cease escape-motivated behaviors. A sample size of 7 mice per group from 3 different dams was used.

2.3. Tissue collection

Tissue samples from each experimental group were collected using a sterile excision process as previously described (Li et al., 2012). Placental and fetal brain tissue was collected from pregnant females and fetuses at E17.5 and snap frozen in liquid nitrogen and