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Full-length Article

Neonatal lipopolysaccharide treatment alters hippocampal neuroinflammation, microglia morphology and anxiety-like behavior in rats selectively bred for an infantile trait

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ABSTRACT

Disruptions in homeostasis, such as the induction of inflammation, occurring during the neonatal period of development often produce changes in the brain, physiology, and behavior that persist through the life span. This study investigated the potential effects that an immune challenge delivered during neonatal development would have on anxiety behavior and stress reactivity later in life within a selectively-bred strain of rat. The rats have been bred for multiple generations to display either high or low anxiety-like phenotypic behavior. On postnatal day (P)3 and P5, male and female neonates were injected with saline or lipopolysaccharide (LPS). Brains were collected from a subset of neonates following injections. At P7, one male and one female per litter were tested for ultrasonic vocalizations (USVs). In adulthood, remaining litter mates were tested on the open field apparatus and the elevated zero maze (EZM) or on the EZM following 3 days of acute stress. Overall, we saw differences between the High and Low lines in neonatal anxiety-like behavior (USVs), neonatal peripheral immune response, adult anxiety-like behavior on the EZM, and adult anxiety-like behavior after stress induction, such that the High line rats display significantly more anxiety-like behavior than the Low line. Furthermore, we observed an effect of neonatal LPS during the neonatal peripheral immune response (e.g., increased inflammatory cytokine expression) and adult anxiety-like behavior on the EZM. We also observed an effect of sex within the anxiety-like behavior of LPS-treated adults exposed to stress paradigm. The combined results shed light on the relationships between neural development, early-life inflammation and anxiety throughout the lifespan.

1. Introduction

The immune system has a critical role in brain function in development, health, and sickness. For example, the primary immunocompetent cells of the brain, microglia, are increasingly implicated in the etiology of many neuropsychiatric disorders (Rico et al., 2010). Immune activation during neonatal development, a time of enormous maturation and increased vulnerability to environmental factors such as a bacterial infection (McGowan, 2015) has been shown empirically to affect both microglial function within the brain (Williamson et al., 2011), adult anxiety-like behavior within the rat model (Sominsky et al., 2012), disease susceptibility, reactivity to stress, and rates of neuropathologies (Bilbo and Schwarz, 2009; Karrow, 2006; Mouihate et al., 2010; Schwarz and Bilbo, 2012; Spencer et al., 2006; Williamson and Bilbo, 2013; Williamson et al., 2011).

Lipopolysaccharide (LPS) exposure during the neonatal period causes a rapid release of pro-inflammatory cytokines and chemokines in the periphery and within the brain, a dramatic increase in circulating corticosterone levels, an increase in TLR signaling molecule expression and other lasting physiological changes (Schwarz and Bilbo, 2012), peaking at about 2 h post-injection (Bilbo et al., 2005; Ortega et al., 2010; Schwarz and Bilbo, 2011; Xu and Ling, 1994). Furthermore, neonatal treatment with LPS leads to HPA axis hyperresponsiveness in adult behavioral paradigms and altered glucocorticoid responsiveness to stress (Granger et al., 1996; Hodgson et al., 2001; Shanks et al., 1995).

Anxiety disorders, depression, schizophrenia, posttraumatic stress disorder, and Rett Syndrome have all been linked to alterations in immune function (Abazyan et al., 2010; Ashwood et al., 2010, 2011; Careaga et al., 2010; Garay and McAllister, 2010; Muller and Ackenheil, 1998; Pace and Heim, 2011; Schwarz and

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Bilbo, 2012; Watanabe et al., 2010). Specifically, anxiety disorders rank among the most common neuropsychiatric disorders diagnosed within the United States, resulting from both familial inheritance and environmental factors (Hanamsagar and Bilbo, 2015; Weissman et al., 1996; Werner et al., 1999; Wickramaratne and Weissman, 2000) and causing clinically significant distress or impairment in social, occupational, or other important areas of functioning (American Psychiatric Association, 2013).

In a model of phenotypic anxiety in the rodent, we utilized two lines of selectively bred rats. Rodents emit ultrasonic vocalizations (USVs) to indicate positive and negative affect and to encourage or discourage prosocial behavior. The vocal response to isolation of rat pups consists of high-frequency ultrasonic vocalizations (USVs) within the range of 40–50 kHz (Allin and Banks, 1972; Brunelli, 2005b). Neonates isolated from their mothers emit USVs as immediate indicators of stress and defense, especially between the ages of postnatal days (P)3-18 (Brunelli, 2005b; Dichter et al., 1996; Hofer, 1996). Upon isolation, the stress response of the pup includes USVs as well as physiological changes within the HPA axis and noradrenergic activity (Brunelli, 2005b). Ultimately, the mother-infant interaction is a behavioral feedback loop that can be strongly modulated by pup USVs.

Selective breeding of rats based on their USVs upon isolation produced two distinct lines: pups with the highest rates of USV emission are the High line and pups with the lowest rates are the Low line (Brunelli, 2005a,b). The phenotypic differences are stable and extend beyond neonatal USVs to adult behavior on the elevated plus maze (EPM) (Dichter et al., 1996), and the Open Field test (Martinez et al., 2015). Based on this stable phenotypic difference between lines, we assessed the effects of neonatal immune challenge with LPS on affective changes in neonates (USVs) and adults (open field, elevated zero maze) as well as molecular changes in the peripheral and central immune and stress responses (e.g., interleukin-1β protein and mRNA expression, corticosterone levels, and microglial morphology) (see Fig. 1 for experimental timeline).

2. Materials & methods

2.1. Animals

Animals were N:NIH Norway rats derived from the 51st generation of High and Low Lines (Zimmerberg et al., 2005) raised in the animal facilities of Williams College. Rats were kept under standard conditions with a 12:12 light:dark cycle (lights on, 6AM) at 22℃ with 50% relative humidity. Rats had unlimited access to food and water. For breeding, females were mated with males from the same line. Pregnant females, determined by the presence of a vaginal plug, were separated from the male and individually housed in plastic cages (45 cm L × 25 cm W × 15 cm H). Dams were kept under the same standard housing conditions and had continuous access to food and water. The day of birth was denoted as P0. Offspring were weaned at 25 days of age, and pair-housed with a same-sex sibling within a plastic cage (45 cm L × 25 cm W × 15 cm H).

All housing and testing procedures were approved by the Williams College Institutional Animal Care and Use Committee.

2.2. Neonatal injections

At P3 and P5, chosen due to their significance in microglia development, the pups in both High and Low lines were separated from the dams and placed into a circular glass dish (20 cm D × 10 cm H) with bedding. All injections were done between 13:00 and 15:00. Their weights and sexes were recorded. Then they received a subcutaneous injection of either endotoxin-free saline (SAL) or *E. coli* – derived lipopolysaccharide (LPS) dissolved in saline (Fig. 1A). All pups in a given litter were given the same treatment and each litter was randomly assigned to a condition. Pups in the SAL group received 0.1 mL of saline, while pups in the LPS group received a dose of 50 μg/kg of LPS. To minimize heat loss, each litter was kept within a dish that rested on a heating pad during the separation from their dam. Following injections, pups were returned to the dams.

2.3. Experiment 1: maternal care assessment & USV testing

2.3.1. Maternal care assessment

Following P3 and P5 injections, pups were returned to the dams. As a measure of maternal care, we recorded the time (s) required for the dam to return all of her pups to the nest. Rather than continuing to disrupt maternal care by continuous observation, this procedure correlates the speed to create a nest with the quality of maternal care.

2.3.2. Ultrasonic vocalization (USV) testing

On P7, 1 male and 1 female pup were randomly selected from each litter (with 6 or more pups) and placed into a circular glass

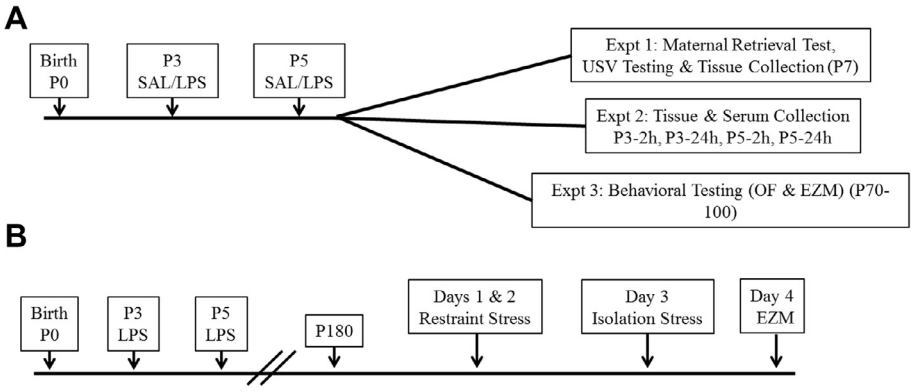

Fig. 1. Experimental timelines for all experiments. (*A*) Experiments 1, 2 and 3 all included rats that experienced neonatal treatment (SAL or LPS) at both P3 and P5. Experiment 1 measured maternal retrieval latencies and USVs in P7 pups. In experiment 2, after saline ($N = 80$) or LPS ($N = 79$) injections, one male and one female from each litter were euthanized at each time point post-injection ($N = 159$). In experiment 3, rats were aged to P70-P100 when adult anxiety-like behavioral assessments were conducted: open field test (OF) and elevated zero maze (EZM). (*B*) In experiment 4, all rats were treated as neonates with LPS at P3 and P5 and aged to P180 at which point they underwent three days of a stress paradigm ($N = 30$) or control handling ($N = 29$). On the fourth day, all rats were tested for anxiety-like behavior on the EZM and immediately euthanized for tissue harvest.