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Overcoming neonatal sickness: Sex-specific effects of sickness on physiology and social behavior

Kristyn E. Sylvia[a,*], Gregory E. Demas[a,b]

[a] Department of Biology, Center for the Integrative Study of Animal Behavior, Indiana University, Bloomington, IN 47405, USA
[b] Program in Neuroscience, Indiana University, Bloomington, IN 47405, USA

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ABSTRACT

Early-life environmental stressors, including sickness, have the potential to disrupt development in ways that could severely impact fitness. Despite what is known about the effects of sickness on reproduction, the precise physiological mechanisms have not yet been determined. The goal of this study was to investigate the effects of a neonatal immune challenge on adult reproductive physiology and opposite-sex social behavior. Male and female Siberian hamster (*Phodopus sungorus*) pups were administered lipopolysaccharide ([LPS]; a cell wall component of gram-negative bacteria) or saline injections on postnatal days 3 and 5 and body mass, food intake, and measures of reproductive maturity were taken throughout development. In adulthood, hamsters were placed in staged mating pairs with reproductively mature individuals of the opposite sex, during which a series of behaviors were scored. We found that although males and females showed no change in food intake, body mass, or reproductive behaviors, LPS-treated females had abnormal estrous cycles and smaller ovaries. Females also showed increased investigation of and increased aggression towards males in a reproductive context. In contrast, LPS-treated males showed no change in any physiological measures, nor did they show any changes in behavior. The present findings demonstrate that females may be more robustly affected by neonatal sickness than males and that these effects could have potential impacts on reproductive success. Collectively, the results of this study can be used to expand upon what is already known about sickness and reproduction, specifically the importance of social behaviors involved in pre-copulation and information necessary to choose the appropriate mate.

1. Introduction

Animals encounter environmental fluctuations throughout their lifetime, and in order to produce appropriate behavioral responses, neural circuits must integrate external stimuli with internal physiology. The hypothalamo-pituitary-gonadal (HPG) axis integrates environmental stimuli and regulates reproductive activity through hypothalamic release of gonadotropin-releasing hormone (GnRH), concomitant release of gonadotropic hormones from the pituitary, and subsequently, sex steroids from the gonads [1,2]. The HPG axis is strongly affected by physiological responses to external stressors, and the timing of environmental stressors is critical in determining the effect they will have on an organism's development. The neonatal period is an extremely sensitive time in the life of an individual. In particular, environmental stressors during this time may increase susceptibility to a range of nervous system disorders (e.g., anxiety, depression, autism, schizophrenia, and learning disabilities) and may result in subtle alterations in the physiological and behavioral response an individual has to subsequent stressors or to new experiences in adulthood [3–5].

Many social behaviors are crucial to successfully attracting a mate and successful reproduction. Reproductive behaviors are extremely diverse, and they are dependent not only species, but also on sex and gonadal steroid levels, as well as time of year [6]. In most female mammals, including Siberian hamsters (*Phodopus sungorus*), lordosis is used to determine the receptivity of a female, and is characterized by a fixed posture accompanied by a dorsiflexion of the back [7]. Male rodents mount shortly after being introduced to a receptive female, followed by intromission and ejaculation [8].

Prior to lordosis and mounting and when first introduced to a conspecific, both sexes will perform vigorous chemoinvestigatory behaviors, allowing them to retrieve the necessary cues for choosing an appropriate mate and copulating [9]. Animals perform nose-to-nose and nose-to-anogenital investigation to identify sex, age, and other characteristics of their conspecifics to determine whether or not the individual is a suitable mate [10,11]. Nose-to-nose sniffing involves the rubbing and passing of the animal's snout through the fur of the conspecific, which often occurs during the early phases of sexual behavior [11,12]. Nose-to-anogenital sniffing involves the rubbing and passing of

* Corresponding author at: Department of Biology, Indiana University, 1001 E. 3rd Street, Bloomington, IN 47405, USA.
E-mail address: ksylvia@indiana.edu (K.E. Sylvia).

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the animal's snout through the fur of the conspecifics anus and genitalia [11,12].

While animals can adjust to environmental stressors during certain periods of their life, some stressors during the early stages of life can have lasting consequences on physiology and behavior. The precise cause of behavioral abnormalities is not completely understood, yet it appears that an inflammatory or sickness response during pregnancy or shortly after birth may play a role. Studies have shown that male mice exposed to prenatal stress have increased expression of cytokines in the hippocampus compared with non-stressed individuals, and they exhibit greater activation of microglia and astrocytes in response to an immune challenge in adulthood [13]. Treatment with lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria, is commonly employed to induce a robust immune response in animals. When LPS is injected into the peritoneal cavity, it enters circulation within fifteen minutes and levels stay elevated for at least 2 h following injection [14]. One way LPS administration mimics the actions of a live bacterial infection is by way of binding to toll-like receptor (TLR)-4, which stimulates the activation of transcription factors and pathways leading to the subsequent release of pro- and anti-inflammatory mediators in the body [15]. These molecules can then act downstream to contribute to the acute-phase response (APR) initiated primarily by hepatocytes, eliciting a sickness response, including but not limited to fever, lethargy, decreased food intake, and enhanced pain [3,16–18].

Although exogenous LPS has been shown to produce increases in mainly pro-inflammatory cytokines (interleukin-1 [IL-1], IL-6, and tumor necrosis factor-alpha [TNF-α]), one study has suggested that treatment with exogenous LPS not only causes an increase in IL-1 (a pro-inflammatory cytokine), but further, they determined that LPS can also stimulate release of the anti-inflammatory cytokine, IL-2, and these two cytokines can differentially influence behavioral responses [19]. For example, rats treated with IL-2 at three weeks of age exhibit enhanced locomotor activity and greater levels of exploration, whereas rats treated with IL-1 at eight weeks of age show an increase in startle response [20], suggesting that the release of these specific cytokines may be the trigger for changes in the amount and duration of particular social behaviors. Work in our lab suggests that maternal immune activation with LPS affects offspring physiological and behavioral development. Specifically, male offspring from LPS-treated dams show greater cortisol response following an intruder encounter and higher frequency of bites during that agonistic encounter when compared with offspring from control-treated dams. Further, these two measures are positively correlated, suggesting the hypothalamo–pituitary–adrenal (HPA) axis may play a role in these behavioral changes [21].

The goals of the present study were to assess the effects of postnatal immune activation on offspring reproductive development and behavior and to evaluate the potential sex differences in response to neonatal treatment. We hypothesized that early postnatal immune activation with LPS would produce changes in both reproductive development and behavior, as well as other social behaviors in both males and females. The results of this study will help to further our understanding of how the neuroendocrine and immune systems interact during early development in male and female Siberian hamsters. More importantly, they will shed light on an important aspect of reproductive behavior that is often missing from the literature; here we focus on the social behaviors particularly important in pre-copulation, which is crucial in determining the optimal mate.

2. Materials and methods

2.1. Animal housing and immune challenge

Male and female hamsters were paired (n = 18 pairs) and housed in a 16:8 light:dark photoperiod, in polypropylene cages (28 × 17 × 12 cm). Ambient temperature was maintained at 20 ± 2C, and relative humidity was maintained at 55 ± 5%. Hamsters were given ad libitum access to tap water and standard laboratory rodent chow (Lab Diet 5001, PMI Nutrition) throughout the experiment. Males of each breeding pair were removed from the breeding cage 16 days after being paired to prevent post-partum pregnancy. Pups remained in their litter until weaning on postnatal day (pnd) 24. On pnd3, half of the litters were given a single intraperitoneal (i.p.) injection (100 μl) of 50 μg/kg of LPS (from *Salmonella enterica* serotype typhimurium, Sigma-Aldrich, St. Louis, MO, USA), suspended in 0.9% sterile saline and the other half of the litters received injections of 0.9% sterile saline. A second injection of LPS or saline was given on pnd5 according to a previously validated protocol, as there is heightened sensitivity of the GnRH pulse generator at these time points [22]. All pups in an individual litter received the same treatment (LPS or saline), and the time for mothers to return to nursing was monitored across all litters. Once injected, pups were monitored and weighed for the remainder of the experiment. Animals were weaned at pnd24 and housed individually for the remainder of the study. At the conclusion of the study, reproductive behavior assays were conducted to determine both pre-copulatory behaviors (e.g., investigation, scent-marking, and aggression) and copulatory behaviors (e.g., lordosis, mounting, and ejaculation). See Fig. 1 for experimental timeline. All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Bloomington Institutional Animal Care and Use Committee (BIACUC) at Indiana University.

2.2. Reproductive physiology

Beginning at pnd25 and once per week thereafter until reproductive maturity (for no > 2 weeks), males (n = 20 saline-treated; n = 26 LPS-treated) were lightly anesthetized with isoflurane, and the length and width of the left testis were measured externally (+/− 0.1 mm) with calipers following previously outlined methods [23–26]. Estimated testis volume (ETV) was calculated as the length × width^2, which is directly correlated with testis mass and spermatogenesis [23,27]. An ETV of 400mm^3 indicates a mass of approximately 200 mg, which is correlated with the critical mass for production of viable spermatids [23,27]. Beginning at pnd25 and every day thereafter until reproductive maturity, all female offspring (n = 24 saline-treated; n = 29 LPS-treated) were monitored daily to determine the time of initial vaginal opening [28]. Female estrous cycles were monitored via vaginal cytology during the 5 consecutive days of behavioral testing (pnd71–75) [29,30]. Vaginal cell samples were obtained via vaginal lavage. Following lavage, samples were transferred to microscope slides, fixed with methanol, and stained with Giemsa. Samples were then evaluated for estrous stage (diestrus, proestrus, estrus, metestrus, and anestrus) under 100 × magnification [29,31,32]. Estrous cycling provides a measure of reproductive functioning, specifically ovarian

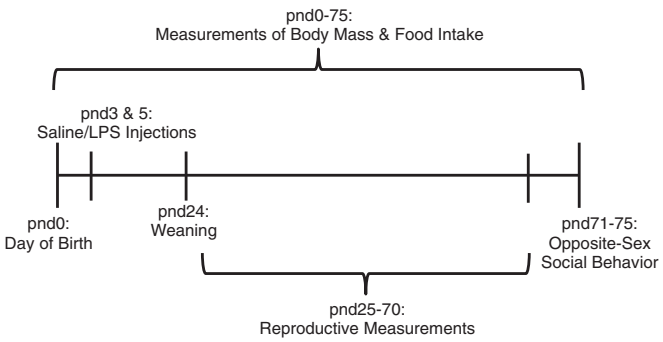

Fig. 1. Experimental timeline demonstrating when treatments were performed and when physiological and behavioral measures were collected. Postnatal day (pnd) 0 represents the time point at which pups were born, and pnd24 represents the time point at which each animal was individually housed for the remainder of the study.

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