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Neonatal Stress Modulates Sickness Behavior

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Abstract

The quality of the early environment, especially during the neonatal period, influences the development of individual differences in resistance to stress and illness in adulthood. A previous study demonstrated that neonatal stress augmented proinflammatory cytokine expression and viral replication in influenza virus-infected adult mice. The goal of the following study was to examine the lifelong effects of neonatal stress on the behavioral response to an immune challenge. Neonatal stress consisted of separating mouse pups from their dams (**maternal separation, MSP**) at critical points of their development. In the first study, pups were separated from the dam daily for 6 hr between postnatal day 1 and 14. As adults, these mice were infected with influenza A/PR8 virus. In a second study, a similar paradigm of MSP was employed, and as adults mice were injected with lipopolysaccharide (LPS) (ip). In a third study pups were separated from the dam for 24 hr on postnatal day 4 or 9. As adults, these mice received ip injections of LPS. In all three studies, changes in body weight, food and sweet solution consumption were examined following immune challenge. As previously described, activation of the immune system using influenza virus infection or LPS administration resulted in sickness behavior that consisted of body weight loss, anorexia and reduced consumption of a sweet solution. Furthermore, neonatal stress induced more rapid kinetics of sickness behavior and augmented several aspects of these symptoms. Together with previous studies, these findings suggest that neonatal stress disrupted the regulation of innate resistance to an immune challenge resulting in enhanced immunological and behavioral responses to immune activation. Thus, long lasting effects of early stress events may be the basis for individual differences in health and susceptibility to disease.

Keywords

Maternal separation; sickness behavior; influenza virus; lipopolysaccharide; mice

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Introduction

Stressful life events in early childhood have been associated with greater risk for mental illness and other chronic diseases in adulthood (Ader and Friedman, 1965; Kaufman et al., 2000; Luecken 1998; Russek et al., 1996; Solomon et al., 1968). Studies have demonstrated that stress early in life elicited long-term changes in multiple neurotransmitter systems and brain structures, and these neurobiological changes may underlie the increased vulnerability later in life (Kaufman et al., 2000). Animal studies support this notion demonstrating an association between neonatal stress, disruption of immune function and increased vulnerability to illness. For instance, premature weaning of rat pups suppressed lymphocyte proliferation, increased opportunistic respiratory tract infections and induced premature deaths (Ackerman et al., 1988). Separation of rat pups from their dams induced a marked increase in the severity of experimental autoimmune encephalomyelitis (EAE) in the adult rats (Teunis et al., 2002), enhanced vulnerability to an experimental model of colitis (Milde et al., 2004) and increased the severity of airway inflammation in an asthma model (Kruschinski et al., 2008). In mice, separation from the dam and premature weaning reduced serum immunoglobulin levels following injection of sheep red blood cells (Michaut et al., 1981). In monkeys, early weaning reduced lymphocyte proliferation in response to B or T cell mitogens (Laudenslager et al., 1985), and lower antibody titer following tetanus toxoid immunization (Coe et al., 1988, Laudenslager et al., 1985).

Extensive study has been conducted to sort out possible mechanisms mediating the effects of early stress. Many of these studies utilized the maternal separation (MSP) model in a variety of animal species. In this model, pups are separated from the dam at critical points of their development and the effects of this stressor are assessed later in life. Studies using this model showed that separation of rat or mouse pups from the dam during the first 2–3 weeks of life augmented the response of the hypothalamic-pituitary-adrenal (HPA) axis to stress in pups and adults (Suchecki and Tufik 1997; van Oers et al., 1998; Plotsky and Meaney, 1993; Schmidt et al., 2003). Maternal separation also resulted in decreased glucocorticoid receptor binding in both the hippocampus and hypothalamus, and increased hypothalamic corticotrophin-releasing hormone (CRH) mRNA, suggesting that maternal separation altered glucocorticoid feedback control (Meaney et al., 1996; Plotsky and Meaney, 1993).

Activation of the HPA axis is an important regulatory mechanism for the inflammatory response to an infectious challenge. Proinflammatory cytokines, secreted from immune cells following infection or injury, activate the HPA axis to release glucocorticoid hormones. These hormones, in turn, act as a negative feedback control and suppress further release of cytokines (Bateman et al., 1989; Blalock, 1994). Since neonatal stress altered HPA reactivity (Suchecki and Tufik 1997; van Oers et al., 1998; Plotsky and Meaney, 1993; Schmidt et al., 2003), it was hypothesized that MSP would also affect host response to an infectious challenge (Avitsur et al., 2006). To examine this hypothesis, MSP mice were infected with influenza A/PR8 virus as adults. The results indicated that MSP augmented several aspects of the response to infection. For instance, infection-induced lung proinflammatory cytokine (interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)- α) mRNA expression was higher in MSP mice compared to controls. These changes in host response to the viral infection were accompanied by an increase in viral replication in lungs

of MSP mice. In addition, influenza-induced corticosterone secretion was blunted in MSP mice, suggesting that the increase in cytokine expression was due to insufficient glucocorticoid feedback control (Avitsur et al., 2006).

Activation of the immune system is typically accompanied by a variety of behavioral changes, collectively termed “sickness behavior” (Hart, 1988; Kent, et al., 1992), such as reduced locomotion and exploratory activity, anorexia and anhedonia, and reduced libido (Avitsur et al., 1999; Dantzer et al., 2007). Proinflammatory cytokines secreted as a part of the inflammatory process have been implicated in mediating sickness behavior (Dantzer et al., 2007).

To sum up, neonatal stress (MSP) increased proinflammatory cytokine levels in response to an influenza virus-infected mice (Avitsur et al., 2006). Elevated levels of proinflammatory cytokines were implicated in sickness behavior during infection (Dantzer et al., 2007). Thus, it may be hypothesized that MSP would also exacerbate the symptoms of sickness behavior in response to an immune challenge. To test this hypothesis, MSP and control mice were infected with influenza virus or injected with lipopolysaccharide (LPS) and the effects of immune activation on symptoms of sickness behavior were measured.

Materials And Methods

Animals

In experiment 1, subjects were male and female offspring of C57BL/6 mice purchased from Charles River Laboratories (Charles River, Inc., Wilmington, MA.). Subjects were born and raised at the Ohio State University Postle Hall vivarium and housed in the Postle Hall facility which is an American Association for the Accreditation of Laboratory Animal Care (AAALAC) accredited facility. In experiments 2 and 3, subjects were offspring of C57BL/6 mice purchased from Harlan Laboratories (Israel). Subjects were born and raised at the Academic College of Tel Aviv-Yaffo (experiment 2) or the Tel Aviv University (experiment 3) animal facilities. All subjects were given free access to food and water and were maintained on a 12-hour light/dark cycle. Animal care procedures were approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee (experiment 1) and the National Israeli Committee of Animal Care and Use (experiments 2 and 3).

Maternal separation (MSP)

Pregnant female C57BL/6 mice were housed individually in standard cages. The age of the pups was determined by checking for births daily, the date of birth was designated as postnatal day 0. Litters were housed with the dam and cages were not disturbed except for MSP procedure. MSP consisted of removing the pups from the home-cage and into a clean standard laboratory cage in sibling groups, while the dam remained in the home cage. In experiments 1 and 2, MSP was performed for 6 hours a day between postnatal day 1 and 14 (Avitsur et al., 2006). In experiment 3, pups were separated from the dam once for 24 hours on postnatal day 4 (MSP4) or 9 (MSP9) (Levine et al., 1991; Schmidt et al., 2002; Stanton et al., 1988; Van Oers et al., 1998). The cages with the pups were placed on a heating pad set at 30–33 °C. Pups had no access to food and water during the separation period. Following

separation pups were returned into the home cages. Observations indicated that following their return to the cages, all pups were retrieved into the nest by the dams within 2–3 minutes. In most cases (>99%) pups did not look weak or debilitated following separation of either 6 or 24 hr. Control litters remained undisturbed in their home cages. Following weaning (at postnatal day 24–26), mice were housed in same-sex groups with their littermates. In all experiments, up to 2 pups per litter were included in the same treatment group.

Virus and Infection

Influenza A/PR8 virus was grown in the allantoic fluid of embryonic eggs and the viral titer was determined by hemagglutination assay. Mice were infected intranasally with 0.05 ml of 0.1 HAU influenza A/PR8 virus diluted in saline. Prior to infection, mice were anesthetized by an intramuscular injection of a Ketamine/Xylazine solution (62.4/3.52 mg/kg) diluted in saline.

LPS administration

Lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 (Sigma, Israel) was injected ip in 0.2 ml sterile saline at 10 (exp. 2) or 20 (exp.3) µg/mouse. Control mice were administered with 0.2 ml sterile saline, ip.

Procedure

Experiment 1: On postnatal day 1, litters were randomly assigned into control and MSP groups. Control litters remained undisturbed in their home cages while MSP litters were separated from the dams for 6 hr/day as between postnatal days 1 and 14 as described above. Further experimental manipulations took place when subjects reached the age of 7–10 weeks. As adults, influenza virus-induced symptoms of illness were assessed. All experimental subjects were individually housed at least 24 hours before the beginning of measurements. Baseline measurements began three days prior to influenza infection. Mice were weighed, returned to their home cages and provided with weighed food, water and saccharine (3M, Sigma) bottles. Mice, food pellets and drinking bottles were weighed again 24 hr later. This procedure was repeated three times on the three days prior to influenza virus infection. On the day of infection, animals were weighed, infected as described above, and following their return to the cage provided with weighed amounts of food, drinking water and saccharin. Similar to the baseline measurements, food and drinking bottles were weighed again every 24 hr for 9 days after infection. In this experiment, males and females were infected and tested at different times. Occasionally a bottle leaked; when this occurred, data for that liquid that day were discarded.

Experiment 2: On postnatal day 1, litters were randomly assigned into control and MSP groups. Control litters remained undisturbed in their home cages while MSP litters were separated from the dams for 6 hr/day between postnatal days 1 and 14 as described above. Further experimental manipulations took place when subjects reached the age of 7–10 weeks. At least 48 hr before the beginning of baseline measurements, subjects were individually housed. Each subject was provided with a sucrose bottle (2% solution in dH₂O, Sigma) for a period of 24 hr. For baseline measurements mice were weighed, returned to their home cage and provided with weighed food, water and sucrose bottles. Food pellets

and drinking bottles were weighed again 2, 4, 6 and 24 hr later. Following the last baseline measurements, animals were injected with either saline or LPS (10 µg/mouse), and supplied with fresh weighed food and drinking bottles. Body weight, food and drinking bottles were weighed again 2, 4, 6 and 24 hr following injections. Occasionally a bottle leaked; when this occurred, data for that liquid at that time point were discarded.

Experiment 3: On postnatal day 1, litters were randomly assigned into control, MSP4 and MSP9 groups. Control litters remained undisturbed in their home cages while MSP litters were separated from the dams once for 24 hr on either postnatal day 4 (MSP4) or 9 (MSP9) as described above. Further experimental manipulations took place when subjects reached the age of 7–10 weeks. Baseline and measurements were conducted as described for experiment 2. Following baseline measurements, animals were injected with either saline or LPS (20 µg/mouse), followed by assessment of changes in body weight, food and liquid consumption as described for experiment 2.

Statistical analysis—To control for individual differences in baseline measurements, data are presented as “difference from baseline” measurements. In experiment 1, body weights measured prior to infection were subtracted from weights measured on each day post infection. Average food and fluid consumption of the three baseline days was calculated. Daily food and fluid consumption were subtracted from these average baseline measures. In experiments 2 and 3, body weights measured prior to saline or LPS injections were subtracted from weights calculated at each time point following injections. Food and fluid consumption were subtracted from baseline measures assessed at the corresponding time interval on baseline day prior to LPS administration. Data describing water consumption are not presented since mice consumed small amounts of water in the presence of the sweet solutions and no differences between experimental groups in water consumption was detected.

In experiment 1, males and females were tested at different times. Thus, data of males and females were analyzed separately. Baseline body weight, food and sucrose consumption were compared using a one-way ANOVA with stress (HC/MSP) as between subject factor. Post infection changes in body weight, food and saccharin consumption data were analyzed using a two factor ANOVA with stress (control/MSP) and day of infection (1–9) as between subject factors. In experiments 2, baseline body weight, food and sucrose consumption were compared using a two-way ANOVA with stress (HC/MSP) and gender (female/male) as between subject factor. Post injections changes in body weight, food and sucrose consumption data for each time point (2, 4, 6, 24 hours post injections) were analyzed using a three factor ANOVA with stress (control/MSP) injections (Saline/LPS) and gender (female/male) as between subject factors. In experiments 3, baseline body weight, food and sucrose consumption were compared using a two-way ANOVA with stress (HC/ MSP4/ MSP9) and gender (female/male) as between subject factor. Post injections changes in body weight, food and sucrose consumption data for each time point were analyzed using a three factor ANOVA with stress (control/MSP4/MSP9), injections (Saline/LPS) and gender (female/male) as between subject factors. Unpaired means comparisons were used to assess

differences between experimental groups where appropriate. Differences were considered significant at $p < 0.05$.

Results

Experiment 1: Effect of MSP on influenza virus-induced sickness behavior

Baseline data are presented in Table 1. Baseline body weight and food consumption were increased by MSP in males ($F(10)=24.7$ and 14.81 for body weight and food consumption, respectively, $p < 0.005$). All other baseline measurements were not affected by MSP ($p > 0.1$). Influenza virus infection resulted in body weight loss, anorexia and reduced saccharin consumption. These effects appeared on days 6–9 post infection as indicated below for the different groups. In addition, MSP modulated several aspects of the effects of influenza virus infection in both females and males.

Females—(Fig 1): ANOVA revealed a significant main effect for influenza virus infection on body weight ($F(9,100)=7.81$, $p < 0.0001$), food ($F(9,100)=18.15$, $p < 0.0001$) and saccharin ($F(9,96)=4.32$, $p < 0.0001$) consumption. Additionally, MSP significantly affected saccharin consumption ($F(1,96)=20.88$, $p < 0.0001$). Significant body weight loss was observed on day 7–8 post infection in controls and 7–9 post infection in MSP females compared to other days of measurements. Significant reduction of food consumption was observed on day 7 post infection in controls and on days 6–9 post infection in MSP indicating that the effect of the virus on food consumption appeared earlier and lasted longer in MSP females compared to controls. Reduced saccharin consumption was observed in controls on day 8 post infection and in MSP on day 7 post infection. In addition, on days 2, 3 and 7 post infection MSP females consumed significantly less saccharine compared to controls.

Males—(Fig. 2): ANOVA revealed a significant effect for influenza virus infection on body weight ($F(9,100)=27.14$, $p < 0.0001$) food ($F(9,100)=28.62$, $p < 0.0001$) and saccharine ($F(9,95)=3.59$, $p < 0.0001$) consumption. Additionally, MSP significantly affected food consumption ($F(1,100)=11.73$, $p < 0.001$). Significant body weight loss was observed on days 7–9 post infection in controls and on days 6–9 post infection in MSP males compared to other days of measurements. Reduced food consumption was observed on days 6–9 post infection in controls and on days 6–8 in MSP. Reduced saccharin consumption was observed on days 3 and 5 post infection in controls and on days 3, 7 and 8 post infection in MSP males. Infection-induced body weight loss was more notable in MSP mice on day 3 post infection compared to controls. Reductions in food and saccharine consumption were significantly more notable in MSP mice compared to controls on day 7 post infection.

Experiment 2: Effect of MSP on LPS-induced sickness behavior

Representative baseline data are presented in Table 2. MSP significantly affected baseline body weight and food consumption ($F(1,56)=7.14$ and 14.46 for body weight and food consumption, respectively, $p < 0.01$). In addition, male baseline body weight was significantly higher than females ($F(1,56)=28.9$, $p < 0.001$). Body weight was increased and food consumption was decreased in female MSP mice compared to controls. All other baseline measurements were not affected by MSP or gender.

LPS administration resulted in body weight loss and anorexia. In females, LPS also reduced sucrose consumption. In addition, MSP modulate the kinetics of the effects of LPS on body weight loss and food consumption.

Body weight loss—ANOVA revealed a significant effect for LPS on body weight at all time points of measurement ($F(1,50)=27.72, 19.13, 50.78$, and 397.12 for 2, 4, 6 and 24 hours, respectively, $p<0.05$). In addition, gender induced a significant effect on body weight at 2, 6 and 24 hr after LPS administration ($F(1,50)=10.49, 7.02$, and 16.21 for 2, 6 and 24 hours, respectively, $p<0.05$). A significant interaction between the effects of gender and MSP on body weight was revealed at 4 hr after injections ($F(1,50)=6.17$, $p<0.05$). A significant interaction between the effects of LPS, MSP and gender on body weight was found at 24 hr after LPS administration ($F(1,50)=8.23$, $p<0.01$).

In control females, LPS induced a significant body weight loss at 2, 6 and 24 hr after administration. In MSP females, LPS significantly reduced body weights at all time points measured. In addition, in saline-injected females body weight of HC was significantly higher than MSP mice 24 hr after injections (Fig. 3A). In control males, LPS significantly reduced body weight at 24 hr after administration. In MSP males, LPS significantly reduced body weight at all time points measured (2–24hr) (Fig. 3D). Comparing body weight loss between males and females revealed that in control LPS-injected mice, body weight were lower than in females compared to males at 2, 4 and 6 hr after LPS administration. In addition, in control saline-injected mice, body weights were higher in females compared to males 24 hr after ip injections.

Food consumption—ANOVA revealed a significant effect for LPS on food consumption at 2, 6 and 24 hr after administration ($F(1,50)=4.17, 17.22$ and 87.3 for 2, 6 and 24 hours, respectively, $p<0.05$). A significant effect for gender on food consumption was revealed at 2, 4 and 24 hr after LPS administration ($F(1,50)=6.1, 4.47$, and 5.76 for 2, 4 and 24 hours, respectively, $p<0.05$). A significant interaction between the effects of gender and MSP on food consumption was revealed at 4 hr after injections ($F(1,50)=5.9$, $p<0.05$).

In control females, LPS significantly reduced food consumption at 24 hr after administration whereas in MSP females this effect appeared both at 6 and 24 hr after LPS administration (Fig. 3B). In control males, LPS reduced food consumption at 24 hr after administration. In MSP male mice, LPS reduced food consumption at all time points beginning at 4 hr after administration. In addition, in LPS injected mice, controls consumed less food than MSP mice at 2 and 24 hr after injections (Fig. 3E). Overall, female mice consumed more food compared to males, and this effect was not modulated by MSP or LPS.

Sucrose consumption—ANOVA revealed a significant effect for LPS on sucrose consumption 24 hr after administration ($F(1,50)=5$, $p<0.05$). MSP induced a significant effect on sucrose consumption 2 hr after LPS administration ($F(1,50)=5.92$, $p<0.05$). Unpaired comparisons revealed that the effect of LPS was similar in control and MSP females (Fig. 3C). In males, LPS had no significant effect on sucrose consumption (Fig. 3F).

Experiment 3: Effect of a single, 24 hr MSP session on LPS-induced sickness behavior

Representative baseline data are presented in Table 3. MSP significantly affected baseline body weight and food consumption ($F(2,92)=10.87$ and 3.57 for body weight and food consumption, respectively, $p<0.05$). In addition, baseline body weight and food consumption were higher in males compared to females ($F(1,92)=195.11$ and 14.65 , for body weight and food consumption, respectively, $p<0.0005$). Baseline body weight was lower in female MSP4 and MSP9 mice compared to controls. All other baseline measurements were not affected by MSP or gender.

LPS administration resulted in body weight loss and anorexia in female and male mice. In addition, LPS reduced sucrose consumption in females. These effects lasted at least 24 hours after injections and MSP modulated the effects of LPS in both females and males.

Body weight loss—ANOVA revealed a significant effect for LPS on body weight at all time points measured ($F(1,86)=15, 47.85, 92.65, 273.26$ for 2, 4, 6 and 24 hours, respectively, $p<0.0005$). MSP significantly affected body weight at 2 and 24 hr after LPS administration ($F(2,86)=5.56, 6.3$ for 2, and 24 hours, respectively, $p<0.05$). A significant effect for gender on body weight loss was revealed at 6 and 24 hr after LPS administration ($F(1,86)=5.1, 4.52$ for 6, and 24 hours, respectively, $p<0.05$). A significant interaction between gender and LPS was detected at 2 hr after administration $F(1,46)=6.22$, $p<0.05$).

In control and MSP9 females, LPS induced body weight loss beginning at 4 hr after administration. In MSP4 females, LPS significantly reduced body weights beginning at 6 hr after administration. In addition, in LPS-injected females body weight of MSP9 was significantly lower than MSP4 mice 24 hr after injections (Fig. 4A). In control males, LPS induced a significant body weight loss 24 hr after administration. In MSP4 and MSP9 males, LPS significantly reduced body weight at all time points measured. Furthermore, in LPS-injected males body weight of MSP4 mice was significantly lower than in controls at 2, 6 and 24 hr after LPS administration. Body weights of MSP9 males injected with LPS were significantly lower than LPS-injected male controls beginning at 2 hr after LPS administration (Fig. 4D). In addition, in saline-injected control group, males lost more weight compared to females.

Food consumption—ANOVA revealed that food consumption was significantly reduced by LPS at all time points measured ($F(1,85)=7.21, 32.17, 16.78, 194.57$ for 2, 4, 6 and 24 hours, respectively, $p<0.01$). MSP induced a significant effect on food consumption at 2 hr after LPS administration ($F(2,85)=3.61$, $p<0.05$). A significant effect for gender on food consumption was detected at 4, 6 and 24 hr after LPS administration ($F(1,85)=5.67, 4.02, 10.51$ for 4, 6 and 24 hours, respectively $p<0.05$).

In control females, LPS significantly reduced food consumption beginning at 6 hr after administration whereas in MSP mice this effect appeared earlier (MSP4 - beginning at 2 hr; MSP9 - beginning at 4 hr after administration). In LPS-injected females, food consumption was lower in MSP4 compared to control mice at 24 hr after injections (Fig. 4B). In control males, LPS reduced food consumption at 6 and 24 hr after administration. In MSP4 and MSP9 male mice, LPS reduced food consumption beginning at 4 hr after administration

(Fig. 4E). Overall, females consumed less food compared to males and this effect was not modulated by MSP or LPS.

Sucrose consumption—ANOVA revealed that sucrose consumption was reduced by LPS at 24 hr after administration ($F(1,86)=16.78$, $p<0.0001$). A significant interaction between the effects of LPS and gender was detected at 24 hr after administration ($F(1,86)=5$, $p<0.05$). Unpaired comparisons revealed that LPS significantly reduced sucrose consumption in control and MSP4 females 24 hr after administration (Fig. 4C). LPS had no significant effect on sucrose consumption in males (Fig. 4E).

Discussion

Early, negative life events have long-lasting consequences on multiple organ systems. Studies have demonstrated that stressful life events early in life resulted in behavioral disturbances, endocrine and immunological disorders and increased susceptibility to disease in humans and a variety of animal species (Coe and Lubach, 2007). A commonly used model for neonatal stress, the maternal separation (MSP) paradigm, reduced resistance to an infectious challenge (Avitsur et al., 2006). MSP induced lasting effects on the immune and endocrine responses to an influenza virus infection in mice. This study showed that MSP increased lung proinflammatory cytokine expression and inhibited glucocorticoid secretion in influenza virus-infected mice (Avitsur et al., 2006).

The present study examined the lifelong changes in the behavioral responses associated with immune activation following neonatal stress. In the first experiment, mouse pups were separated from the dams for six hours a day from postnatal day 1 to 14. As adults, these mice were infected with a low dose of influenza virus and their behavioral response was examined. In agreement with previous reports (Swiergiel and Dunn 1999, Swiergiel et al., 1997), influenza virus infection induced sickness behavior that consisted of body weight loss, anorexia and reduced consumption of sweet solutions. This behavioral response developed relatively slowly and the peak of the response appeared between day 6–9 post infection in most groups. The data further showed that MSP resulted in more rapid kinetics and increased severity of influenza virus-induced sickness behavior. For instance, influenza virus-induced anorexia was observed in control females on day 7, whereas in MSP females anorexia was observed earlier – on day 6 post infection, and lasted longer – until day 9 post infection. Similarly, the peak of the effect of influenza virus infection on saccharine consumption was observed earlier (day 7 post infection) in MSP females compared to controls (day 8 post infection). In MSP males, infection-induced suppression of food and saccharin consumption during the peak of the response (day 7 post infection), was significantly more notable in MSP mice compared to controls. These findings suggest that neonatal stress had lasting effects not only on the endocrine and immunological aspects of the influenza virus infection (Avitsur et al., 2006), but also on the behavioral symptoms associated with disease.

To further explore the long lasting effects of neonatal stress on sickness behavior a second study was conducted. In this study, adult control and MSP mice were injected with LPS. Administration of LPS is known to induce sickness behavior and a rapid release of

proinflammatory cytokines (Dantzer et al., 2007). In agreement with previous reports, the present data demonstrated that LPS induced body weight loss, anorexia and reduced consumption of sweet solution within a few hours (Avitsur and Yirmiya 1999; Dantzer et al., 2007). Furthermore, MSP induced more rapid kinetics of the effect of LPS on food consumption. In control males and females, LPS significantly reduced food consumption 24 hr after administration. In MSP mice, anorexia appeared earlier (6 hr post administration in females and 4 hr in males). A similar effect, although smaller, was demonstrated in the effect of LPS on body weight change in males. LPS significantly reduced body weight in controls 24 hrs after administration, whereas in MSP the effect of LPS was significant at all time points measured. Interestingly, in males the effect of LPS on food consumption was more pronounced in controls compared to MSP at 24 hr, perhaps indicating a faster return to baseline. These findings support the conclusion that neonatal stress modulated the behavioral symptoms associated with immune activation and further suggest that this effect was not specific to influenza infection but may accompany other types of immune challenges, particularly those associated with proinflammatory cytokine responses.

The undisturbed, healthy development of the HPA axis is essential for the development of adequate host resistance to disease (Bateman et al., 1989; Blalock, 1994). Thus, changes in the HPA reactivity could result in inadequate control of the immune response and altered susceptibility to disease. One of the characteristics of the developing neuroendocrine stress system in rats and mice is the stress hypo-responsive period (SHRP). This period is mainly characterized by low basal levels of corticosterone and the inability of mild stressors to induce an increase in corticosterone and ACTH (Dallman, 2000; Sapolsky and Meaney, 1986; Schmidt et al., 2002). This period lasts from right after birth until postnatal day 12 in the mouse (Schmidt et al., 2003). The SHRP is followed by a period of enhanced basal and stress-induced corticosterone levels, gradually developing into an adult-like response (Schmidt et al., 2003). The development of the HPA stress response is under maternal regulation and is disrupted by separation of the pups from the dams (Levine et al., 1991; Schmidt et al., 2003; Stanton et al., 1988). In the first two studies described in this report, as well as in our previous studies (Avitsur et al., 2006), MSP was performed daily during the SHRP. These studies demonstrated that MSP during this period induced lasting changes in the immunological, endocrine and behavioral responses to immune challenges. This MSP paradigm, however, did not provide information regarding the specific stage of the HPA development that was critical for the effects of MSP. The last experiment included in this report began to examine the critical neonatal period for the development of MSP-induced changes in host response to an immune challenge. In this study, mice were separated from the dam for 24 hours on postnatal day 4 (MSP4) or 9 (MSP9) (Levine et al., 1991; Schmidt et al., 2002; Stanton et al., 1988; Van Oers et al., 1998). As adults, mice were injected with LPS. Similar to the previous study, the results demonstrated that LPS induced sickness behavior that lasted at least 24 hours after administration. In addition, the kinetics of this effect was more rapid and in some cases the severity of the symptoms was enhanced in MSP mice. LPS-induced anorexia appeared in MSP mice (2/4 hr post injection) earlier than in controls (6 hr post injection) in both females and males. In addition, LPS induced anorexia was significantly more severe in MSP males compared to controls and LPS-induced body weight loss appeared in MSP mice (2hr post injection) earlier than in controls (6 hr post

injection). Furthermore, these results demonstrated minor differences between MSP4 and MSP9 in some cases, while in most instances MSP4 and MSP9 paradigms induced similar effects on behavior. This finding suggests that postnatal days 4 and 9 are similarly important to the development of LPS-induced symptoms of disease. To thoroughly identifying the stage of HPA development in which MSP-induced changes occurred, additional studies including systematical alterations in the timing of the separation procedure are needed.

The response to immune activation is modulated by complex interactions between genes and environment. Gender and gonadal hormones are important factors that were shown to affect disease susceptibility. Higher prevalence and intensity of infections was found in males compared to females (Zuk and McKean, 1996), while females exhibit greater predisposition for autoimmune disorders and allergies (Grossman, 1985; McCruden and Stimson, 1991). Studies have shown that certain aspects of the behavioral response to disease were affected by gender in rats (Avitsur and Yirmiya, 1999). In the present study, the response to LPS was similar in male and female mice in most cases. However, the data suggest that neonatal stress affected sickness behavior in a gender-dependent manner. Comparing the effect of LPS on body weight loss showed more rapid kinetics and more severe response in females compared to males in home-cage controls. In contrast, in MSP mice faster kinetics and increased severity of the response on body weight loss was apparent in males. This finding suggests that gender is an important factor in modulating the response to neonatal stress and should be taken into consideration in studying the complex interactions modulating the response to immune activation.

The mechanism underlying the effects of neonatal stress on sickness behavior in the adult mouse was not examined in the present study. However, a previous report may suggest a role for proinflammatory cytokines in this effect. As was mentioned above, MSP augmented lung proinflammatory cytokine (interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)- α) mRNA expression in influenza virus infected MSP mice compared to controls (Avitsur et al., 2006). Proinflammatory cytokines were implicated in sickness behavior following a variety of infectious challenges (Dantzer et al., 2007). Together with the present findings, it may be suggested that MSP-induced increase in proinflammatory cytokines secretion was involved in the alterations in the kinetics and severity of sickness behavior in MSP mice. Moreover, corticosterone levels were lower in influenza virus-infected MSP mice compared to controls (Avitsur et al., 2006). Glucocorticoid hormones serve as a regulatory mechanism to prevent over activation of the immune response to infection (Bailey et al., 2003). Amongst its many regulatory roles, corticosterone was suggested to play a protective role in limiting the expression of sickness behavior following an immune challenge (Johnson et al., 1996). It may therefore be suggested that the blunted corticosterone secretion in influenza virus-infected MSP mice (Avitsur et al., 2006) may have been involved in the changes in the severity and kinetics of sickness behavior demonstrated in the present study. Additional studies are needed to examine the mechanisms of the effects of MSP on the response to immune challenge and the possible role of proinflammatory cytokines and glucocorticoid hormones in these effects.

An important consideration in interpreting these data is that separation of pups from the dam at an early age may not necessarily be a negative life event. In nature, the dam would

occasionally leave the pups to search for food for limited periods of time. This provides the pups with an opportunity to explore their environment and may enrich their early experiences. According to this notion, the forced uninterrupted contact between the dam and the pups in laboratory conditions may be disadvantage to the development of the pups. In line with this view, it was demonstrated that repeated short daily separation of rat pups from the dam reduced their endocrine response to stress (Meaney et al 1989). Due to the complexity of the experiments included in this report the effect of short durations of maternal separation on illness behavior were not examined. However, based on these report it may be speculated that certain events occurring during early childhood may be beneficial for the host response to pathogen invasion and may reduce the behavioral response to illness.

During the early postnatal period, mice are dependent upon the mother for warmth, food, and bodily waste excretion. Subjecting pups to maternal separation during this period resulted in lasting changes in stress reactivity accompanied by alterations in various organ systems (Millstein and Holmes 2007; Coe and Lubach, 2007). In humans, many studies have demonstrated the importance of family function and early life events as predictors of health in adulthood. These studies showed that the quality of family life influenced the development of individual differences in vulnerability to illness throughout life (reviewed by Cameron et al., 2005). For instance, a prospective study examined the incidence of acute respiratory infections in healthy infants in the Tbilisi Infant's House and a control group under normal maternal care. This study showed increased disease incidence and severity of infection in maternal deprived infants (Gogberashvili 2006). Such studies have given rise to models of "Stress diathesis" as explanations for the relation between the quality of early life and health in adulthood. According to these models, early environment, particularly parental care, influences the development of neural systems that underlie the expression of behavioral and endocrine responses to stress. Thus, adversity or decreased quality of parental investment increases the magnitude of emotional, autonomic and HPA responses to stress in adulthood. The augmented activation of neural and hormonal responses to stress was suggested to promote illness (reviewed by Cameron et al., 2005). The present study further contributes to the understanding of the association between the quality of the early environment and the development of individual differences in host response to disease.

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Fig. 1. Effect of an influenza virus infection on body weight (A) food (B) and saccharin consumption (C) in control (HC) and maternally separated (MSP) female mice (n=6/group). To control for individual differences in baseline measurements, all data are presented as “difference from baseline” measurements. Data are presented as mean $\pm$ SEM in grams (gr). * Significantly different from corresponding control group (same day post infection) ($p < 0.05$).

Fig. 2.

Effect of an influenza virus infection on body weight (A) food (B) and saccharin consumption (C) in control (HC) and maternally separated (MSP) male mice (n=6/group). To control for individual differences in baseline measurements, all data are presented as “difference from baseline” measurements. Data are presented as mean $\pm$ SEM in grams (gr). * Significantly different from corresponding control group (same day post infection) (p<0.05).

Fig. 3.

Effect of lipopolysaccharide (**LPS**, 10 μ g/mouse) or saline (**sal**) injections on body weight (**A**-females, **D**-males) food (**B**-females, **E**-males) and sucrose consumption (**C**-females, **F**-males) in home-cage control (**HC**) and maternally separated (**MSP**) female mice ($n=5-9$ /group). To control for individual differences in baseline measurements, all data are presented as “difference from baseline” measurements. Data are presented as mean $\pm$ SEM in grams (**gr**). * Significantly different from corresponding saline-injected control group

(same neonatal treatment and time post injection) ($p < 0.05$). ^A Significantly different from corresponding HC group (same ip injection and time post injection) ($p < 0.05$).

Fig. 4. Effect of lipopolysaccharide (LPS, 20 μ g/mouse) or saline (sal) injections on body weight (A-females, D-males) food (B-females, E-males) and sucrose consumption (C-females, F-males) in home-cage control (HC), maternally separated on postnatal day 4 (MSP4) and maternally separated on postnatal day 9 (MSP9) female mice (n=6–9/group). To control for individual differences in baseline measurements, all data are presented as “difference from baseline” measurements. Data are presented as mean $\pm$ SEM in grams (gr). * Significantly different from corresponding saline-injected control group (same neonatal treatment and

time post injection) ($p < 0.05$). ^A Significantly different from corresponding HC group (same ip injection and time post injection) ($p < 0.05$). ^B Significantly different from corresponding MSP4 group (same ip injection and time post injection) ($p < 0.05$).

Table 1

Baseline values of body weight, food and saccharine consumption measured prior to influenza virus infection in home cage (HC) and maternally separated (MSP) male and female mice (Exp.1).

		Body Weight	Food	Saccharine
Female	HC	22.7 (0.8)	3.9 (0.25)	16.4 (1.4)
	MSP	22.1 (0.36)	3.8 (0.09)	19 (0.9)
Male	HC	19.5 (0.52)	3.4 (0.04)	13.6 (0.91)
	MSP	23.2 (0.52) *	4 (0.14) *	13.5 (0.72)

Values presented as mean (S.E.M). Baseline body weight (grams) was measured prior to infection. 24 hr- food and saccharine consumption was measured for 3 consecutive days. The values presented are the average (S.E.M) of the three baseline days.

* Significantly different from corresponding HC group ($p < 0.05$).

Table 2

Baseline values of body weight, food and saccharine consumption measured prior to saline or LPS administration in home cage (HC) and maternally separated (MSP) male and female mice (Exp. 2).

		Body Weight	Food	Sucrose
Female	HC	18.5 (0.76)	4.4 (0.34)	23.9 (2.63)
	MSP	20.9 (0.66) *	3 (0.24) *	26.1 (2.9)
Male	HC	23.4 (0.57)	3.9 (0.25)	26.6 (4.65)
	MSP	25.9 (1.16)	3.4 (0.14)	27.1 (4.01)

Values presented as mean (S.E.M). Baseline body weight (grams) was measured prior to ip injections. Food and saccharine consumption were measured for 24 hours.

* Significantly different from corresponding HC group ($p < 0.05$).

Table 3

Baseline values of body weight, food and saccharine consumption measured prior to saline or LPS administration in home cage (HC), maternally separated on postnatal day 4 (MSP4) and on day 9 (MSP9) male and female mice (Exp. 3).

		Body Weight	Food	Sucrose
Female	HC	23.7 (0.44)	3.7 (0.32)	15.7 (1)
	MSP4	20.4 (0.84) *	4.4 (0.22)	16 (1.23)
	MSP9	21.7 (0.53) *	4.4 (0.27)	15.4 (0.88)
Male	HC	30.8 (0.68)	2.8 (0.28)	16.3 (1.2)
	MSP4	28.8 (0.84)	3.6 (0.37)	15.9 (1.1)
	MSP9	27.8 (0.33)	3.3 (0.18)	14.8 (0.98)

Values presented as mean (S.E.M). Baseline body weight (grams) was measured prior to ip injections. Food and saccharine consumption were measured for 24 hours.

* Significantly different from corresponding HC group ($p < 0.05$).