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Social interactions alter proinflammatory cytokine gene expression and behavior following endotoxin administration

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Abstract

Sick animals display a constellation of behaviors, including anhedonia, anorexia, and reduced social interactions. Acute infection eliminates female mating behavior, but fails to attenuate mating behavior in male rats. These results have been attributed to the different reproductive strategies and parental investment of the two sexes. Males putatively suppress the symptoms of infection in order to "deceive" females into mating. We sought to investigate the mechanisms responsible for this suppression. Adult male CD-1 mice were treated with lipopolysaccharide (LPS; a component of bacterial cell walls; 400 μg/kg), then paired 2 h later with a receptive female or juvenile male or remained isolated. Blood samples and brains of the males were collected 3 h post-LPS; hypothalamic interleukin-1 (*IL-1*) and tumor necrosis factor-α (*TNFα*) gene expression was measured using RT-PCR. Contrary to our prediction, exposure to a female *increased* hypothalamic *IL-1* and *TNFα* gene expression. LPS treatment significantly decreased testosterone and increased corticosterone secretions. Social interactions altered absolute corticosterone concentrations in saline-injected animals only. In order to determine whether increased production of hypothalamic cytokines reflected increased severity of sickness responses, body temperature was monitored in a second group of mice implanted with telemetric transmitters. Body mass, food intake, and consumption of sweetened condensed milk (a highly favored food) were also monitored in these mice for 72 h post-injection. LPS injections reduced milk intake, an effect that was modulated by social interactions; however, fever was unaltered relative to isolated animals. These results suggest that social interactions can adjust behavioral responses to infection although the ultimate cause of this adjustment remains unspecified.

Keywords: Sickness behavior; Lipopolysaccharide; Cytokines; IL-1; TNF; Social interaction

1. Introduction

Animals treated with lipopolysaccharide (LPS; endotoxin), a component of gram negative bacterial cell walls, display a coordinated suite of physiological and behavioral responses (Exton, 1997). The behavioral sequelae of endotoxin-induced infection are particularly salient and include lethargy, anorexia, adipsia, anhedonia, and a reduction in social interactions (Hart, 1988). These responses collectively termed 'sickness behavior,' along with the induction of fever, are thought to be part of a coordinated, adaptive effort to aid in recovery from infection (Hart, 1988; Kent et al., 1992). The primary mediators of the sickness response are the proinflammatory cytokines, interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNFα) (Dantzer, 2001; Kent et al., 1992).

LPS injections in the periphery induce cytokine gene expression both in the periphery and in the central nervous system. Although the precise mechanisms remain under investigation, peripheral cytokines are thought to induce central production of cytokines via vagal activation (Ek et al., 1998; Goehler et al., 1999) and by diffusion into circumventricular brain regions

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(Quan et al., 1998). Expression of cytokines in the brain appears to underlie the behavioral effects of peripheral LPS administration (Laye et al., 2000). In the brain, cytokines are largely expressed in microglia and perivascular and meningeal macrophages (van Dam et al., 1992).

Although sickness behavior has been conceptualized as part of an adaptive strategy to overcome infection, sickness responses are plastic (Aubert et al., 1997b; Bilbo et al., 2002). For instance, lactating mice treated with LPS did not build nests when their cages were housed at room temperature. However, LPS-treated dams constructed nests that were equivalent to saline-injected dams when the ambient temperature was reduced to 6 °C, a temperature that jeopardized pup survival (Aubert et al., 1997a). LPS treatment reduced food hoarding only slightly in rats that received all their food from hoarding relative to those given supplemental food. However, immediate food intake was reduced to a similar level in both groups (Aubert et al., 1997b).

Male and female mating behavior is differentially affected by LPS administration. LPS prevents the preovulatory LH surge in female rats and also eliminates mating behavior in ovariectomized, hormonally primed females (Rivier and Vale, 1990) via the induction of prostaglandins (Avitsur et al., 1999). In males, however, LPS fails to disrupt mating at all but the highest doses (Yirmiya et al., 1995) despite suppressing testosterone production (Guo et al., 1990; Turnbull and Rivier, 1997). Moreover, this sex difference is specific to mating because LPS affects behavior similarly between males and females in other behavioral paradigms not related to mating (Yirmiya et al., 1995). Indeed, male rats will suppress the symptoms of their infection, putatively to 'deceive' females into mating (Avitsur and Yirmiya, 1999).

Although the effects of LPS or cytokine administration on social behaviors have been extensively documented, the role of social interactions in modulating the sickness response has received relatively little attention. The present study was designed to investigate the physiological mechanisms underlying the suppression of sickness behavior. In particular, we hypothesized that exposure to a receptive female, but not a juvenile male, would attenuate proinflammatory gene expression in the hypothalamus of male mice. In contrast to our prediction, exposure to females dramatically increased hypothalamic cytokine gene expression and altered the behavioral response to LPS.

2. Materials and methods

2.1. Animals

The Ohio State University Institutional Lab Animal Care and Use Committee approved all animal protocols in accordance with National Institutes of Health guidelines. Adult (8–12 weeks of age) male CD-1 mice (*Mus musculus*) were individually housed in polycarbonate cages (28 × 17 × 12 cm) with ad libitum access to food and water throughout the experiment. Animals were housed in colony rooms maintained on a 14:10 light–dark cycle (lights on at 00 h EST) and temperatures of 20 ± 4 °C and relative humidity of 50 ± 10%. After allowing 1 week to acclimate to the laboratory environment, all animals were exposed to an intact female for a period of 8 days to allow them to become sexually experienced.

Stimulus females were bilaterally ovariectomized under isoflurane anesthesia. The females were hormonally primed by injecting 50 μg of estradiol benzoate (i.p. in 0.1 ml of corn oil) 48 h prior to the start of the experiment, followed by 500 μg of progesterone (i.p. in 0.1 ml of corn oil) on the morning of testing. Approximately 1 h before the beginning of the social interactions, hormonally primed females were exposed to a sexually experienced male stud in order to ensure that they were in behavioral estrus. The pairs were observed live and females were not used unless they exhibited the lordosis reflex at least three times during the testing sessions. Juvenile males were CD-1 weanlings (21–28 days old) bred in our laboratory.

2.2. Experiment 1

Mice were randomly assigned to one of six experimental groups: (1) saline/isolated, (2) saline/+juvenile male, (3) saline/+female, (4) LPS/isolated, (5) LPS/+juvenile male and, (6) LPS/+female ($n = 10$/group). At 14 h (1 h before lights out) on the day of testing animals were injected i.p. with either sterile saline or 400 μg/kg of lipopolysaccharide (LPS; Sigma, *Escherichia coli*, serotype 026:B6, Sigma Chemical, St. Louis, MO) suspended in 0.1 ml saline. Animals were then returned to their home cages.

At 16 h males were moved to a testing room illuminated with photographic red light. The food and water was removed and the designated stimulus animal (e.g., juvenile male or hormonally primed female) was introduced into the experimental male's home cage. All social interactions were videotaped for subsequent behavioral analysis.

2.2.1. Tissue collection

Following 1 h of social interaction, experimental males were cervically dislocated and trunk blood was collected. Blood was allowed to clot at room temperature for at least 30 min, clots were removed, blood was spun at 2500 rpm for 30 min at 4 °C, and serum was stored at −70 °C until assayed for testosterone and corticosterone concentrations. In addition, brains were removed using aseptic techniques and the hypothalami

were quickly dissected out, placed in sterile tubes, and frozen on dry ice.

2.2.2. Behavioral scoring

Videotapes were scored using The Observer 5.0 Software (Noldus, Leesburg, VA) system by an observer unaware of the experimental conditions. For interactions with juvenile males, tapes were scored for the duration of time the experimental male spent sniffing the juvenile, auto- and allogrooming, as well as the amount of time spent immobile. Immobility was operationally defined as $\geqslant 3$ consecutive seconds without observable motion. Interactions with females were scored identically to those with juvenile males with one exception: male–female interactions were also scored for mating behaviors. The frequency of mounts, intromissions, and ejaculations, as well as the latency to the first incidence of each of these events was recorded (Quinlan et al., 1989).

2.2.3. RIA procedures

Serum corticosterone (MP Biomedicals, Costa Mesa, CA) and testosterone (Diagnostic Systems Laboratories, Webster, TX) concentrations were assayed using double-antibody ^{125}I kits. The assays were conducted following the guidelines set by the manufacturer. These kits are highly specific and crossreactivity with other steroids was less than 1% for corticosterone and 3.5% for testosterone. Intra-assay variance was less than 10% for both assays and minimum detection thresholds were 5 ng/ml for corticosterone and 0.19 ng/ml for testosterone.

2.2.4. Real-time PCR

Total RNA was extracted from $\leqslant 30$ mg of individual hypothalami using a homogenizer (Ultra-Turrax T8, IKA Works, Wilmington, NC) and an RNeasy Mini Kit according to manufacturer's protocol (Qiagen, Valencia CA). Extracted RNA was suspended in 30 μl RNase-free water and RNA concentration was determined by spectrophotometer (SmartSpec 3000, Bio-Rad, Hercules, CA). All RNA samples were stored at −70 °C until further analysis.

Primers and probes (Overbergh et al., 1999) were synthesized as follows, with probes labeled with 6-FAM (fluorescent dye) and MGB (non-fluorescent quencher) at the 5′ and 3′ ends, respectively: *IL-1β* forward 5′-CAACCAACAAGTGATATTCTCCATG-3′, *IL-1β* reverse 5′-AGATCCACACTCTCAGCTGCA-3′, *IL-1β* probe 5′-CTGTGTAATGAAAGACGGCACACC CACC-3′; *Tnfα* forward 5′-CATCTTCTCAAAATTCG AGTGACAA-3′, *Tnfα* reverse 5′-GGGAGTAGA CAAGGTACAACCC-3′, *Tnfα* probe 5′-CACGTCG TAGCAAACCACCAAGTGA-3′. A TaqMan 18S Ribosomal RNA primer and probe set (labeled with VIC fluorescent dye; Applied Biosystems, Foster City, CA) were used as the control gene for relative quantification. Amplification was performed on an ABI 7000 Sequencing Detection System by using Taqman Universal PCR Master Mix. The universal two-step RT-PCR cycling conditions used were: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative gene expression of individual samples run in duplicate was calculated by comparison to a relative standard curve.

2.3. Experiment 2

Males were randomly assigned to one of four experimental groups: (1) saline/isolated ($n = 13$), (2) saline/+female ($n = 9$), (3) LPS/isolated ($n = 10$), or (4) LPS/+female ($n = 14$). Prior to treatment, animals in all groups were exposed to sweetened condensed milk (Kroger Brand; 1:1 with distilled water) for 6 h each night (16–22 h) on two consecutive nights. Mice were then implanted intraperitoneally with a radiotelemetric transmitter (PDT-4000; Minimitter, Bend, OR) under isoflurane anesthesia and allowed 48 h to recover. Cages were then placed on receivers and connected to a personal computer. Emitted temperature frequencies were collected in 10 min intervals (bins) and converted to temperatures based on preprogrammed calibration curves from each transmitter. Experiment 2 was conducted in four iterations with all groups represented in each.

For the two nights prior to injection baseline body temperature and milk intake was recorded. For 6 h each night (16–22 h) the food and water bottles were removed and a bottle containing 10 ml of sweetened condensed milk was introduced into the cage. Total milk intake was recorded at the end of each 6 h session by subtracting the amount remaining from the original volume. On the day of injection (5 days after surgery) animals were injected with either LPS or saline at 13 h and then exposed to a female from 15 to 16 h or left isolated. Milk intake was monitored on the day of injection and the subsequent day. In addition, ad lib food consumption, body weight, and temperature were recorded through the conclusion of the experiment. Mice were euthanized 72 h post-injections.

2.4. Data analysis

Results of Experiment 1 were analyzed by two-factor (injection type × social interaction) ANOVAs. ANOVAs were followed by Tukey HSD post hoc tests. The fever responses in Experiment 2 were analyzed with a repeated measures ANOVA with time post-injection as a within subject variable and treatment and social condition as between subject variables. The repeated measures ANOVA was followed by multiple one-way ANOVAs at each time point. All mean differences were considered significant if $p < .05$.

3. Results

3.1. Experiment 1

3.1.1. Social behavior

LPS reduced the amount of time that experimental males spent actively interacting with both types of stimulus animals (i.e., the juvenile male or female) (Fig. 1). A two-factor ANOVA (treatment ×social interaction) revealed an overall main effect of both treatment [$F(1,33) = 20.015$, $p < .0001$] and of type of social interaction [$F(2,33) = 3.839$, $p < .01$] and an interaction between the two variables [$F(1,33) = 3.108$, $p < .05$]. The interaction suggests that although social investigation of females by LPS-injected males was significantly reduced, this effect was not as pronounced as the decrease in investigation in the LPS-injected males exposed to juveniles. LPS significantly reduced the time engaged in both auto- [$F(1,33)$, $F = 6.767$, $p < .0001$] and allogrooming [$F(1,33)$, $F = 35.411$, $p < .0001$] and increased the time spent immobile [$F(1,33)$, $F = 26.838$, $p < .0001$]. Social interaction type did not modulate the effects of LPS on these behaviors (see Table 1).

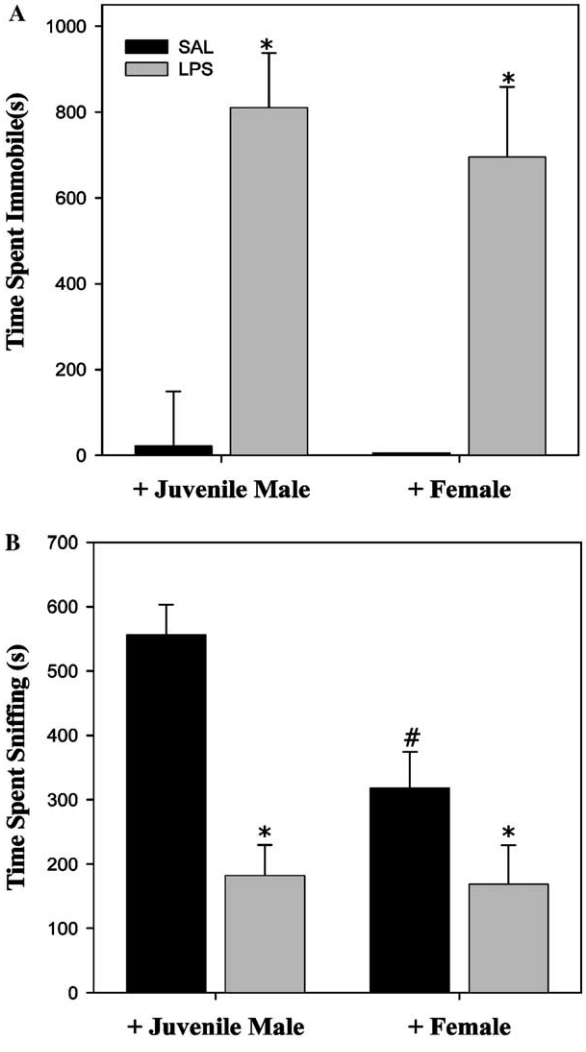

Fig. 1. Effects of LPS on mean (±SEM) time spent immobile during 1 h social interaction (A); mean time spent sniffing stimulus animal (juvenile male or female) (B). *Significantly different from saline-injected animals; #significantly different from saline + juvenile male group; $p < .05$.

Table 1
Effects of LPS administration on behavior in the 1 h social interaction

Behavior	Social interaction	
	Juvenile male	Female
Sniffing		
Saline	556.13 ± 44.08	318.12 ± 52.68#
LPS	182.32 ± 50.29*	168.31 ± 64.92*
Allogrooming		
Saline	271.31 ± 59.90	370.92 ± 71.60
LPS	31.73 ± 12.24*	20.65 ± 15.8*
Autogrooming		
Saline	71.45 ± 60.46	197.94 ± 72.26
LPS	1.73 ± 3.49*	11.95 ± 4.5*
Immobile		
Saline	21.66 ± 12.18	0
LPS	810.09 ± 182.15*	695.06 ± 235.1*
Mounts		
Saline	—	1.67 ± .77
LPS	—	0
Intromissions		
Saline	—	0.444 ± .239
LPS	—	0

Data are presented as mean values (±SEM).
* Significantly different from saline-injected group.
# Significantly different from saline/juvenile male group; $p < .05$.

3.1.2. Testosterone

LPS administration dramatically reduced testosterone concentrations relative to saline-injected animals [$F(1,60) = 38.815$, $p < .0001$; Fig. 2A]. However, social interaction did not significantly alter the testosterone concentrations in any group ($p > .05$).

3.1.3. Corticosterone

LPS administration dramatically increased serum corticosterone concentrations [$F(1,60) = 208.358$, $p < .0001$]. There was also an overall main effect of social interaction type [$F(2,60) = 3.916$, $p < .05$] and an interaction between the two variables [$F(5,60) = 11.608$ $p < .0001$; Fig. 2B]. However, this effect was driven by the saline-injected animals as social interaction did not alter absolute corticosterone concentrations in LPS-injected animals ($p > .05$). However, the relative increase of corticosterone concentration in the LPS-injected animals compared to the saline-injected animals exposed to females was less than in juvenile-exposed animals. Within the saline-injected animals, each social interaction type differed significantly from the other two in regards to corticosterone concentrations ($p < .0001$).

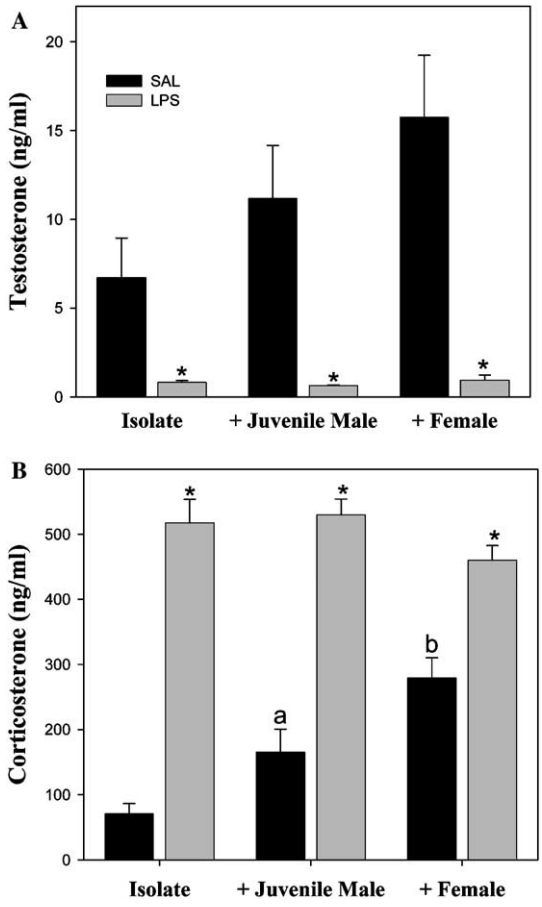

Fig. 2. Effects of LPS administration on serum testosterone (ng/ml; mean ± SEM) in males following social interactions (A), corticosterone (ng/ml) (B). LPS dramatically decreased testosterone concentrations and increased corticosterone concentrations. *Significantly different from saline-injected animals; [a]significantly different from saline/isolate and saline/female groups; and [b]significantly different from saline/isolate and saline/juvenile male groups; $p < .05$.

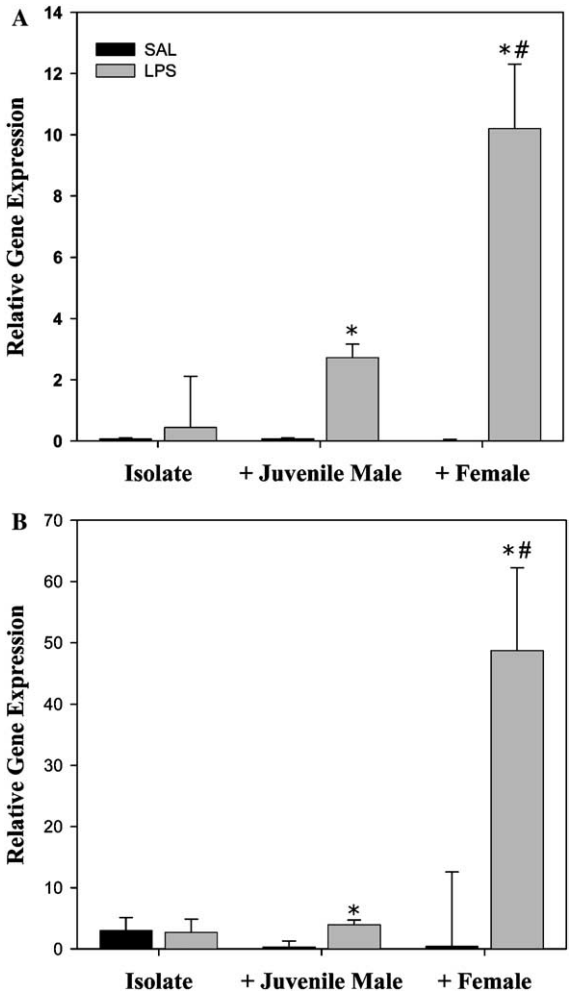

Fig. 3. Effect of social interactions on hypothalamic *IL-1β* (A) and *TNFα* (B) gene expression relative to *18S* rRNA (mean ± SEM). Interaction with a hormonally primed female dramatically increased expression of both proinflammatory cytokine genes in LPS-injected animals. *Significantly greater than saline-injected animals of the same condition; [#]significantly greater than all other groups; $p < .05$.

3.1.4. Cytokine gene expression

Relative gene expression of both *IL-1* [$F(1,37) = 15.967$, $p < .0001$] and *TNF* [$F(1,51) = 3.381$, $p < .05$] were increased in LPS-injected animals as compared to those injected with saline. Social interactions (juvenile male or female) also increased gene expression of *IL-1* [$F(2,37) = 6.464$, $p < .005$] and *TNF* [$F(2,51) = 5.137$, $p < .01$, see Fig. 3] relative to isolated control groups. In addition, there was a significant interaction between the two variables (injection type and social interaction) for the expression of both cytokines ($p < .005$ in both cases). Post hoc analyses indicate that animals that engaged in either type of social interaction had significantly elevated levels of gene expression as compared to saline-injected animals in the same social condition, but isolated LPS-injected animals did not differ from their saline controls ($p > .05$). Finally, within the LPS-injected group both gene transcripts were elevated in the juvenile-exposed group male as compared to isolated animals, but female-exposed animals were higher than all other groups ($p < .001$).

3.2. Experiment 2

3.2.1. Sweetened condensed milk intake

LPS administration reduced intake of sweetened condensed milk and this effect was modulated by exposure to a female. Specifically, both LPS [$F(1,43) = 48.615$, $p < .0001$] and exposure to a female [$F(1,43) = 6.738$, $p < .01$] significantly reduced milk intake on the day of injection. There was not a significant interaction between the two variables on milk intake. Post hoc analysis revealed that the main effect of behavior was mediated by the significant decrease in intake within the LPS group by mice exposed to females ($p < .05$, see Fig. 4). On

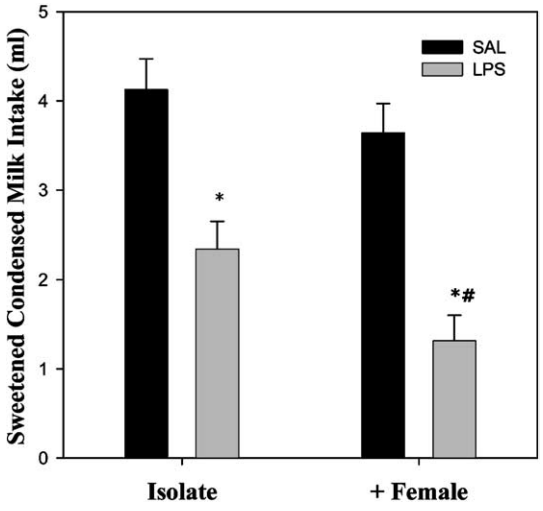

Fig. 4. Mean (±SEM) sweetened condensed milk intake following LPS administration and social interactions. LPS decreased milk intake to a greater extent in males exposed to females than those that were isolated. *Significantly different from saline-injected animals; #significantly lower than LPS/isolate group; $p < .05$.

the day subsequent to injection, milk intake did not differ between groups ($p > .05$).

3.2.2. Body weight and food intake

LPS administration reduced bodyweight [$F(1,43) = 21.058$, $p < .0001$] but not food intake on the day of injection. Neither effect was modulated by social interactions. Body weight had not returned to pre-injection levels by the end of the day following injections in the LPS groups ($p > .05$ relative to baseline).

3.2.3. Fever

LPS significantly increased body temperature regardless of the type of social interaction. A repeated measures ANOVA yielded a main effect of both treatment [$F(1,43) = 11.284$, $p < .002$] and of social interaction [($F(1,43) = 5.094$, $p < .05$]. The onset of fever was not apparent until the beginning of the inactive phase (lights on; 24 h; see Fig. 5). In any case, social interactions did not appear to modulate the effect of endotoxin administration on fever.

4. Discussion

Sickness behavior is part of a coordinated response to assist individuals in overcoming infection (Dantzer, 2001; Hart, 1988). However, in certain behavioral paradigms the symptoms of infection can be suppressed in favor of engaging in other adaptive activities (e.g., Aubert et al., 1997a). LPS administration did not alter mating behavior in male rats; furthermore, male rats appeared qualitatively to suppress the symptoms of sickness in the presence of females (Avitsur and Yirmiya, 1999; Yirmiya et al., 1995). Therefore, we predicted that exposure to a female would attenuate the symptoms of sickness behavior in male mice. Additionally, because the hypothalamus mediates the behavioral and febrile responses to LPS, we predicted that hypothalamic cytokine gene expression would be reduced following exposure to a female. In contrast to our prediction, we observed a potentiation rather than reduction in gene expression and an alteration in the severity of the sickness responses.

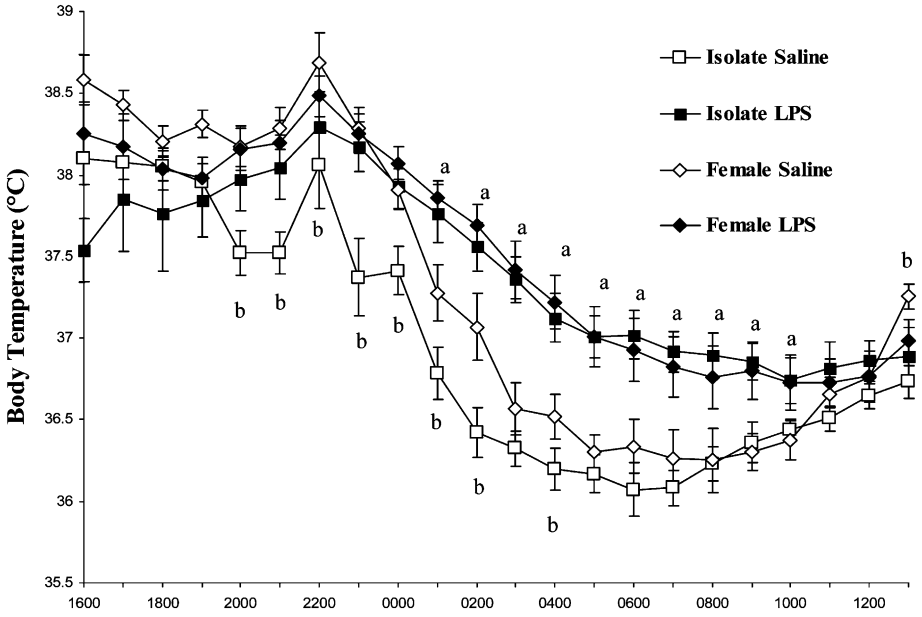

Fig. 5. LPS induced fever in males of both social conditions. Data are presented as means (±SEM) [a]Significant difference between all LPS-injected animals and saline-injected animals; and [b]significant difference between saline-injected animals; $p > .05$.

Consistent with previous findings, LPS administration strongly activated the hypothalamic–pituitary–adrenal (HPA) axis (Tilders et al., 1994; Turnbull and Rivier, 1995) as indicated by elevated corticosterone concentrations that did not differ among LPS-injected animals. The two types of social stimuli differentially affected corticosterone in saline-injected mice. Although corticosterone values are equivalently high in all LPS-treated mice, corticosterone concentrations were significantly elevated by female exposure, reducing the percentage difference between the groups. This may simply reflect a ceiling effect for corticosterone secretion. Glucocorticoid secretion is potently activated by cytokines that feed back to inhibit cytokine gene expression via classic neuroendocrine feedback (Turnbull and Rivier, 1995; Goujon et al., 1997). However, the possibility that elevated cytokine gene expression is the result of a stress response stemming from an intruder animal entering the cage is unlikely because stress tends to reduce the expression of proinflammatory cytokines and also because corticosterone concentrations did not differ among the LPS-injected animals.

Species differences between mice and rats likely underlie the LPS-induced elimination of mating behavior in this study (Yirmiya et al., 1995). However, the generally similar life history strategies between mice and rats make this species difference somewhat unexpected. The increased cytokine gene expression in response to social stimuli likely represents one of two possibilities. First, acute exposure to a female during infection causes dysregulation of the negative feedback mechanisms of the cytokine network. In mice, glucocorticoid insensitivity of splenocytes can be induced by chronic social disruption stress, which in turn increases expression of cytokine genes and a higher mortality following LPS administration (Quan et al., 2001). On the other hand, it is possible that mice have evolved a different strategy than rats to cope with contemporaneous infection and presence of a sexually receptive female. Importantly, social interactions did not alter fever responses to LPS, suggesting that regulatory processes were not disrupted. Mice may have accelerated the time course of the sickness response to hasten recovery and enable them to engage in mating when the infection has been cleared. Another possibility is that reduced social interactions may require more cytokine gene product than other aspects of the sickness response (e.g., fever and lethargy). Thus, in the presence of a receptive female, the increased cytokine expression may be necessary to counteract the motivation to engage in social behaviors. However, in the absence of a conspecific, increased cytokine gene expression could be both unnecessary and have deleterious effects. Additional studies are necessary to tease apart both the ultimate and proximate causes underlying this effect.

The dramatically increased expression of cytokine genes in female-exposed and LPS-injected animals occurred without significantly altering the behavioral response. Males exposed to females exhibited attenuated sickness responses as indicated by the smaller reduction in social investigation compared to animals exposed to juvenile males. Taken together, these results suggest that the behavioral responsiveness of the brain to cytokines may have been altered by female exposure.

Adult mice will vigorously investigate an unfamiliar conspecific introduced into their cages (Fishkin and Winslow, 1997) depending on the age, sex, and reproductive condition of the stimulus animal (Winslow and Camacho, 1995). This behavior is robust and highly motivated in rodents (Gheusi et al., 1994). LPS decreases the amount of time an adult will spend investigating a juvenile. As such, social investigation of a juvenile male has been used extensively as a tool for assessing the magnitude of sickness responses (Bluthe et al., 1994; Fishkin and Winslow, 1997). The present results suggest that exposure to a juvenile male can alter the expression of proinflammatory cytokine genes relative to isolated mice. Therefore, the use of this paradigm to measure sickness behavior may be inappropriate because it may alter the sickness responses under investigation.

This study reversed the traditional paradigm of inducing sickness behavior with LPS or recombinant cytokines, and then observing the effects on behavior. Our results support the concept that sickness responses can be modified by the immediate social environment. A putative relationship has been suggested between immunological activation and a variety of clinical psychiatric disorders (Capuron and Dantzer, 2003; Cleeland et al., 2003; O'Brien et al., 2004). Further, the role of more long-term social factors in other aspects of immunity has been well established (Detillion et al., 2004; Kiecolt-Glaser, 1999). Therefore, it follows that a better understanding of the mechanisms underlying the interactions between the social environment and sickness responses could have important clinical and functional implications.

Acknowledgments

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