Published in final edited form as:

Physiol Behav. 2017 December 01; 182: 128–136. doi:10.1016/j.physbeh.2017.10.011.

Assessment of social behavior directed toward sick partners and its relation to central cytokine expression in rats

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Abstract

Acute illness not only reduces the expression of social behavior by sick rodents, but can also lead to avoidance responses when detected by healthy, would-be social partners. When healthy animals interact with a sick partner, an intriguing question arises: does exposure to a sick conspecific elicit an anticipatory immune response that would facilitate defense against future infection? To address this question, healthy adult male Sprague-Dawley rats (N=64) were given a brief social interaction (30 min) with a partner that was either sick (250 µg/kg injection with lipopolysaccharide [LPS] 3 h prior to test) or healthy (sterile saline injection). During this exposure, social behavior directed toward the healthy or sick conspecific was measured. Additionally, the impact of housing condition was assessed, with rats group- or isolate-housed. Immediately after social interaction, brains were harvested for cytokine assessments within socially-relevant brain structures (olfactory bulb, amygdala, hippocampus and PVN). As expected, behavioral results demonstrated that (i) there was a robust suppression of social interaction directed against sick conspecifics; and (ii) isolate-housing generally increased social behavior. Furthermore, examination of central cytokine expression in healthy experimental subjects revealed a modest increase in TNF- α in rats that interacted with a sick social partner, but only in the olfactory bulb. Among the LPS-injected partners, expected increases in IL-1 β , IL-6, and TNF- α expression were observed across all brain sites. Moreover, IL-1 β and IL-6 expression was exacerbated in LPS-injected partners that interacted with isolate-housed experimental subjects. Together, these data replicate and extend our prior work showing that healthy rats avoid sick conspecifics, and provide preliminary evidence for an anticipatory cytokine response when rats are exposed to a sick partner. These data also provide new evidence to suggest that recent housing history potentially modulates cytokine responses evoked by LPS.

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Keywords

lipopolysaccharide; sickness behavior; social behavior; olfactory bulb; cytokine expression; isolate-housing

Introduction

Positive social interactions have the capacity to attenuate an individual's reactivity to stressors [1–3] and reduce the risk for various diseases [4]. In fact, social interaction is often utilized as a robust and extremely sensitive index of health status [5, 6]. On the other hand, the absence of positive social interactions is highly correlated with physical and mental illness [7], and is typically utilized as a key criterion for multiple mental health diagnoses (DSM V). Thus, there is substantial interest in elucidating the behavioral, neural, and physiological parameters that govern social processes. In young rodents, social behavior typically consists of social play and active investigation of a conspecific through sniffing and social contact behavior [8–11]. Using these rodent models, social processes have been shown to be easily disrupted by a wide variety of circumstances, including stress challenges [12, 13], natural aging [14] and acute illness [5, 6, 15, 16].

Interestingly, the health status of conspecifics can also have a profound influence on the social behavior exhibited toward a social partner. For example, several studies have demonstrated suppression of social behavior directed toward sick conspecifics [17]. Recent studies have also shown that when normal mice are housed with tumor-bearing conspecifics, the “healthy” mice develop immunological aberrations indicative of allergic hypersensitivity [18]. Very little is known, however, regarding the impact of interaction with a sick social partner on expression of central expression of pro-inflammatory cytokines and other aspects of neuroimmune function. Thus, one primary goal of this Experiment was to investigate whether exposure to a sick social partner would stimulate brain expression of cytokines - small proteins, including chemokines, interleukins, and lymphokines, which are known to be important for initiation of the immune response to infection or damage (see Figure 1). To do this, experimental animals briefly interacted with a social partner that was either healthy or rendered sick via acute injection with bacterial endotoxin, otherwise known as lipopolysaccharide (LPS; a component of the cell wall of gram-negative bacteria). Stimulation of the immune system by systemic LPS administration results in a coordinated set of adaptive behavioral responses, collectively termed “sickness behavior” [19–21]. Sickness behavior constitutes an array of symptoms exhibited by animals during the course of an acute infection, including fever, hypoactivity, reduced food and water intake, and reduced social and exploratory behavior [16, 22, 23]. When injected peripherally, LPS also induced marked elevations in proinflammatory cytokine expression by 2 h post-injection throughout the body including in the hypothalamus, hippocampus, cortex, and pituitary that accompany the behavioral sickness response [16, 24, 25]. Thus, LPS injection has been shown to be a reliable method for inducing acute illness in rodent models without the enduring influence of introducing a replicating pathogen. Importantly, illness responses were verified in the present study through examination of cytokine expression in brain and through plasma measures of cytokines.

Indeed, as mentioned above, rodents actively avoid conspecifics that have become ill and that exhibit sickness behaviors and odors. This protective response, which consists of the detection of sickness cues and subsequent avoidance of their source, has also been observed in humans and has been termed the “behavioral immune system” (for review see [26]). Avoidance of sick conspecifics is presumably an adaptive evolutionary response that is designed to limit the spread of infection. From this standpoint, one intriguing question is whether exposure to a sick social partner might also elicit an anticipatory immune response in the healthy subject as a means of priming the immune system to more rapidly and/or effectively respond to future antigen exposure/infection. It was hypothesized then, that when placed in contact with a sick partner, a healthy rat would not only avoid interaction with the sick conspecific, but would also exhibit significant increases in CNS cytokine expression. Brain structures that are instrumental to the processing of socially-relevant and stress-related stimuli were examined in the present experiment and included the olfactory bulb, hippocampus, amygdala, and paraventricular nucleus of the hypothalamus (PVN).

Whereas the health status of a social partner has been shown to significantly influence behavior directed toward that conspecific, there is substantial evidence to suggest that the social environment (i.e. housing conditions) of the experimental test subject also determines the level of social behavior expressed in subsequent, discrete tests of social interaction [27, 28]. For example, both short-term (not more than 24 h) and long-term (several days to several weeks) social deprivation have often been used to increase the frequency of social behavior exhibited during social testing [14, 28]. On the other hand, prolonged social deprivation can have seriously detrimental effects, particularly when the social deprivation is incurred during critical developmental epochs [29–31]. Moreover, social housing is not always beneficial, as enhanced conflict within the social group can produce deleterious consequences in most species (e.g., [32, 33]). Hence, in the current study, social housing conditions of the healthy experimental rats were manipulated in order to assess the impact of recent social experience on subsequent social engagement with a sick or healthy partner. To the extent that sickness-like states represent a shift in motivational state [15], it is possible that enhanced social investigation induced by brief social isolation would over-ride the tendency to avoid social interaction with a sick social partner [34]. More specifically, it was hypothesized that healthy experimental rats that were pair-housed prior to social interaction testing would interact less with sick social partners that had been injected with LPS, but that this avoidance would be attenuated in subjects that were more socially motivated due to social deprivation (via short-term isolate-housing) prior to social interaction (see Figure 1).

Materials and Methods

Subjects

Adult male Sprague-Dawley rats purchased from Harlan Laboratories (Indianapolis, IN) were pair-housed in standard transparent Plexiglas cages with access to food and water ad libitum. Rats were shipped at weights of 225–250 g and allowed to acclimate until achieving weights of 300–350 g. Rats were provided wooden chew sticks for enrichment and colony conditions were maintained at 22±1°C with 12:12 light–dark cycle (lights on 0700). Upon arrival, rats were given a minimum of 1 week to acclimate to our colony conditions. Healthy

experimental rats were then either pair- or isolate-housed for 5 days prior to the onset of experimentation as this length of social deprivation has previously been shown to enhance social interaction [34], while their future social partners maintained under pair-housed conditions. Two days before the start of the experiment, all rats were handled for 3–5 min on each day. Experimental procedures used in the current study were approved by the Institutional Animal Care and Use Committee at Binghamton University, and all animals were treated in accordance with PHS policy.

Drug administration

Initially diluted in sterile, pyrogen-free saline (0.9%) LPS was stored in aliquots of 1 mg/ml at -20°C until the time of experimentation. LPS (*E. coli* serotype 0111:B4) was purchased from Sigma Chemical Co. (St. Louis, MO). Fresh aliquots were then thawed and further diluted in sterile, pyrogen-free saline to the desired concentration. A dose of 250 $\mu\text{g/kg}$ was selected and injected intraperitoneally (i.p.) at 1 ml/kg, as this dose has been previously shown to induce robust sickness-like behaviors [35, 36], as well as increases in central and peripheral cytokine expression [37].

Social Interaction Test

Social interaction tests were conducted during the light period of the circadian cycle (09:00 to 13:00) and under dim light (15–20 lux). Test chambers ($45 \times 30 \times 30$ cm) were composed of Plexiglas® (Binghamton Plate Glass, Binghamton, NY) and were divided into two equally sized compartments by a clear Plexiglas® partition, with an aperture (9×7 cm) in the bottom center of the divider allowing movement of rats between compartments. A layer of clean wood shavings was placed in the bottom of the apparatus. After each test, chambers were cleaned with water, dried with paper towels, and given fresh bedding. Sessions were recorded by a video camera (Sony HDR-CX240) and later analyzed by a trained experimenter that was blind to the experimental condition of each animal. During testing, four behavioral measures exhibited by the healthy experimental subjects were scored and analyzed: crossovers (movement of the experimental subject's body, not including the tail, through the aperture) either toward or away from the social partner, social investigation (sniffing of any part of the body of the partner), and social contact (the sum of crawling over and under the partner and social grooming) (e.g., see [34]).

Experimental procedure

Experimental Test Subjects—A total of 32 healthy experimental rats were used for the 2 (Social Partner Status: healthy vs. sick) $\times$ 2 (Housing Condition: pair- vs. isolate-housed) between subjects factorial design of this study. These young adult male rats (290–315 g) were either maintained in a pair-housed situation in the homecage prior to test, or were isolate-housed for 5 consecutive days prior to social testing. On the day prior to the social interaction test, experimental rats were first pre-exposed to the social interaction chamber for 30 min. This pre-exposure session served to familiarize rats to the social context and encourage more social interaction on the test day (e.g., see [28, 34]). On the following day, half of the animals were placed into the social interaction chamber with another healthy adult male rat (previously injected with pyrogen-free saline), or were exposed to a sick

conspecific (given 250 µg/kg LPS 3 h before test). The social interaction test was 30 min in duration, with animals killed immediately after the test for collection of trunk blood and brains.

Social Partners—Another set of 32 young adult males (315–345 g) served as the social partners to the experimental rats. These social partners were also pre-exposed to the test chamber on the day prior to social interaction testing. Three hours before the 30-min social interaction test, partners were given an i.p. injection of either pyrogen-free saline (healthy social partners) or LPS (sick social partners). Each animal experienced only one social interaction test, and thus partners were not used on multiple occasions. Similar to the experimental rats, social partners were also killed immediately after testing for collection of blood and brains. Given that these animals were ordered from a breeding facility, we cannot be absolutely certain that social partners and experimental rats were entirely unrelated and unfamiliar to each other. Upon arrival, however, extreme care was taken to ensure that experimental rats and social partners did not interact with each other prior to testing.

Tissue and blood collection

Brain tissue was harvested after rapid decapitation and trunk blood was collected into EDTA-coated glass collection tubes (BD Vacutainers, VWR cat. No, VT6450, Radnor, PA). Whole brains were removed immediately after decapitation, were flash frozen in 2-methylbutane (isopentane; cat. No, 78-78-4, Sigma Chemical Co., St. Louis, MO), and then stored at –80 °C. Plasma was separated in a refrigerated centrifuge [4°C, rpm] and frozen at –20 °C until time of assay. Key structures were identified with a brain atlas [38]. To isolate regions of interest, brains were coronally sliced on a cryostat. Bilateral tissue punches (1.2 – 2.0 × 1.0 – 2.0 mm) were then taken from structures of interest [olfactory bulb, hippocampus, PVN, and amygdala; see Figure 1] and immediately stored at –80 °C.

Measurement of corticosterone (CORT)

Plasma CORT concentrations were measured using commercially available Enzyme Immuno Assay (EIA) kits (Enzo Life Sciences; Farmingdale, NY). Samples were processed and analyzed in accordance to the manufacturer instructions, with the exception that samples were heat-inactivated to denature endogenous corticosteroid binding globulin (CBG), rather than through enzymatic degradation. To do this, samples were immersed in a 75 °C water bath for 60 min. Prior assays conducted within our laboratory have demonstrated that this procedure produces superior denaturation of CBG compared to the steroid displacement step provided in the kit (unpublished observations, as discussed in [39, 40]). This assay has been shown to have a sensitivity of 27.0 pg/ml, with an inter-assay coefficient of 5.40%.

Tissue processing

Each tissue sample was placed in a 2.0 ml Eppendorf tube with 500 µL TrizolR RNA® reagent and a 5 mm stainless steel bead. Tissue was then homogenized using a Qiagen TissueLyser II™ (Qiagen, Valencia, CA) for 2–4 min at 20 Hz to ensure thorough homogenization of samples. Total cellular RNA was extracted from tissue using Qiagen RNeasy Mini kits according to the manufacturer's instructions. RNA was separated from the supernatant through chloroform extraction performed at 12,000 g for 15 min at 4 °C. Equal

volume of 70% ethanol was added to the collected RNA and purified through RNeasy mini columns. Columns were washed and eluted with 30 μ L of RNase-free water (warmed to 65 °C). RNA yield and purity was determined using the NanoDrop 2000 spectrophotometer (NanoDrop, Wilmington, DE). RNA was stored at –80 °C prior to cDNA synthesis.

Real-time RT-PCR

Synthesis of cDNA was performed on 0.3–1.0 μ g of normalized total RNA from each sample using the QuantiTect Reverse Transcription kit (Cat No. 205313, Qiagen, Valencia, CA), which included a DNase treatment step. All cDNA was stored at –20 °C until further processing. Probed cDNA amplification was performed in a 20 μ L reaction consisting of 10 μ L IQ SYBR Green supermix (Bio-Rad Laboratories), 0.1 μ L forward and reverse primer, 2 μ L cDNA template, and 8.8 μ L ribonuclease-free water. Samples were run in triplicate in a 384-well plate (BioRad Laboratories) using the BioRad CFX 384 Real Time System C1000 Thermal Cycler (BioRad Laboratories). Relative gene expression was quantified using the 2^{–CT} method [41], with target gene expression calculated relative to the reference gene, β -actin (see [42] for primer sequences). Before analyses of target genes, reference gene expression was first separately analyzed in each structure/tissue compartment to ensure stability across treatment groups.

In the four brain structures of interest, several immune-related factors were analyzed. Interleukin-1 β , IL-6, and TNF- α were measured, as these three cytokines have been shown to be potentially activated in brain in response to systemic infection (e.g., [43]), with other non-immune challenges such as stress (e.g., [44, 45]) or ethanol exposure (e.g., [42, 46]) also altering central expression of these targets. Additionally, expression of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (I κ B α) was also assessed, as this is considered a reporter of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) activation. Finally, expression of c-Fos provided a marker of general neural activation in these brain structures, such that the specificity of cytokine alterations could be indirectly addressed.

Analysis of plasma cytokines in social partners

Circulating plasma cytokines were measured in the social partners injected with either saline or LPS using a commercially available multiplex kit (Bioplex Rat Cytokine 12-Plex Assay panel, BioRad Inc., Hercules, CA). Samples were quantified using a Magpix system (BioRad, Hercules, CA) for the simultaneous detection and quantification of cytokines. The multiplex kit included 12 cytokine and other immune-related peptides: IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, GM-CSF, INF- γ and TNF- α . Total protein was measured using a commercially available Pierce™ bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockford, IL), with plasma cytokine data expressed in pg/100 μ g of total protein.

Data analysis

All analyses were conducted in Statistica 7 (StatSoft; Tulsa, OK) using analyses of variance procedures (MANOVA and ANOVA), as described below. Data from healthy experimental subjects are first presented. In these animals, behavior during the social interaction test and

plasma CORT concentrations were analyzed using a between subjects 2 (Social Partner: healthy vs. sick) $\times$ 2 (Housing Condition: pair- vs. isolate-housed) factorial ANOVA. In order to control for multiple comparisons and minimize the probability of Type 1 error, brain cytokine expression data were first analyzed for experimental rats using MANOVA for each brain region (including all target genes for that structure as within-subjects dependent variables), with follow-up ANOVAs as appropriate (previously described in [45]).

Subsequent to data obtained from healthy experimental rats, brain cytokine, plasma cytokine, and plasma CORT data from social partners are presented. These data were analyzed separately using a between subjects 2 (Drug: saline vs. LPS) $\times$ 2 (Social Condition: pair-housed vs. isolated-housed experimental partner) factorial design.

For all analyses, an α -level of < 0.05 was used. Additionally, any data points that were greater than 2 Standard Deviations above or below the mean were considered outliers, and were excluded from analyses. Finally, Fisher's least significant difference (LSD) post hoc tests were used to determine the locus of significant differences between groups when a significant main effect or interaction term was observed.

Results

Social behavior, central cytokine expression, and plasma CORT in healthy experimental rats

Suppression of social interaction directed against sick social partners (Figure 2)—Main effects of Social Partner Status (healthy vs. sick) for frequency of sniffing [$F(1, 24) = 8.82, p < 0.01$], crosses toward [$F(1, 24) = 16.00, p < 0.001$], and crosses away [$F(1, 24) = 6.32, p < 0.05$], revealed that interaction with a sick social partner significantly suppressed social behavior directed toward that partner, as well as general activity during the 30 min social interaction test (Figure 2A, D, and C, respectively). When social contact was examined, 5 days of social isolation significantly increased contact with a healthy social partner, however this social-stimulatory effect of isolate-housing was blunted by interaction with a sick partner [significant Social Partner Status $\times$ Housing Condition interaction: $F(1, 24) = 5.15, p < 0.05$; Figure 2B].

Central cytokine expression in healthy experimental rats exposed to a sick social partner (Figure 3, Supplemental Figure 1)—Neither interaction with a sick partner, nor Housing Condition, significantly impacted central cytokine or c-Fos expression in PVN, amygdala, or hippocampus in healthy experimental rats (Supplemental Figure 1). In the olfactory bulb, however, a significant effect of Social Partner Status was observed, with the MANOVA indicating that, when all cytokines were loaded as dependent variables, healthy experimental rats that interacted with a sick social partner exhibiting modest, but significant, cytokine elevations in this structure [$F(1, 27) = 5.31, p < 0.05$] (See Figure 3). This effect of a sick social partner was found to be specific to TNF- α expression within the olfactory bulb [$F(1, 27) = 5.31, p < 0.05$], with follow-up 2×2 ANOVAs for each target gene indicating that neither Social Partner Status, nor Housing Condition, influenced gene expression of any other targets examined within the olfactory bulb.

Plasma CORT concentrations in healthy experimental animals (Table 1)—

Immediately after the 30 min social interaction test, analysis of plasma CORT in healthy experimental rats revealed no significant effects of Social Partner Status or Housing Condition on circulating CORT.

Assessment of cytokine expression and plasma CORT in social partner rats

Central gene expression in social partner rats after injection and social interaction testing (Figure 4)—In the social partner rats, MANOVA results for the olfactory bulb, PVN, amygdala, and hippocampus indicated that LPS administration markedly increased cytokine expression in all four of these brain regions [MANOVA main effect of Drug Condition for the olfactory bulb: $F(5, 19) = 25.99, p < 0.001$; PVN: $F(5, 23) = 236.90, p < 0.001$; amygdala: $F(5, 21) = 51.82, p < 0.001$; and HPC: $F(5, 23) = 70.19, p < 0.001$]. Subsequent 2×2 ANOVAs were conducted on each gene target within each structure to determine the locus of these significant effects of LPS in the social partners. Overall, an LPS-induced increase in IL-1 β , IL-6, TNF- α , and I κ B α expression was observed in the olfactory bulb, PVN, and amygdala [main effect of Drug Condition in *olfactory bulb*: IL-1, $F(1, 27) = 40.19, p < 0.001$; IL-6, $F(1, 26) = 31.35, p < 0.001$; TNF- α , $F(1, 26) = 85.29, p < 0.001$; I κ B α , $F(1, 26) = 8.62, p < 0.01$; *PVN*: IL-1, $F(1, 27) = 45.27, p < 0.001$; IL-6, $F(1, 28) = 39.73, p < 0.001$; TNF- α , $F(1, 28) = 136.11, p < 0.001$; I κ B α , $F(1, 28) = 93.44, p < 0.001$; *amygdala*: IL-1, $F(1, 26) = 30.38, p < 0.001$; IL-6, $F(1, 25) = 58.62, p < 0.001$; TNF- α , $F(1, 26) = 80.12, p < 0.001$; I κ B α , $F(1, 26) = 73.18, p < 0.001$; *hippocampus*: IL-1, $F(1, 28) = 78.84, p < 0.001$; IL-6, $F(1, 27) = 65.96, p < 0.001$; TNF α , $F(1, 27) = 112.19, p < 0.001$; I κ B α , $F(1, 27) = 33.60, p < 0.001$]. Whereas c-Fos in the PVN was also increased by LPS injection [$F(1, 27) = 27.99, p < 0.001$], in contrast, social partners given LPS exhibited general decreases in c-Fos expression in the olfactory bulb, amygdala, and hippocampus [*olfactory bulb*: $F(1, 26) = 10.44, p < 0.01$; *amygdala*: $F(1, 26) = 4.62, p < 0.05$; *hippocampus*: $F(1, 28) = 29.94, p < 0.001$]. Social interaction with a healthy experimental animal that was either pair- or isolate-housed did not further alter central gene expression in the olfactory bulb, PVN or amygdala.

According to the MANOVA, the hippocampus was uniquely sensitive to an interaction between Drug Condition and Experimental Partner Housing Condition [$F(5, 23) = 2.82, p < 0.05$]. More specifically, 2×2 ANOVAs for each target in the hippocampus revealed that LPS-induced increases in IL-1 β and I κ B α expression in this region were further potentiated when sick social partners interacted with a healthy experimental animal that had been isolate-housed [for IL-1 β : $F(1, 28) = 5.22, p < 0.05$; for I κ B α : $F(1, 27) = 4.31, p < 0.05$]. Additionally, for IL-6 in the hippocampus, there was main effect of the experimental partners' housing condition [$F(1, 27) = 5.54, p < 0.03$], with interaction with an isolate-housed experimental partner leading to greater IL-6 expression relative to social partners that interacted with a pair-housed experimental animal.

Plasma CORT concentrations—Plasma CORT (Table 1) was analyzed in social partners immediately after the social interaction test. No significant differences in CORT were observed between vehicle-injected and LPS-injected social partners at the end of behavioral testing.

Plasma cytokines—Plasma cytokines concentrations (Table 2) measured in the social partners revealed that LPS administration elicited a significant increase in IL-6 when compared to vehicle-injected controls [$F(1, 28)=5.14, p < 0.05$]. No other significant effects of Drug Condition or Experimental Partner Housing Condition were observed, although non-significant trends for decreased plasma IL-1 ($p=0.080$), IL-4 ($p=0.089$), and IL-12 p70 ($p=0.069$) were seen after LPS injection.

Discussion

Using a social interaction paradigm in which animals can fully engage in social contact, the current study demonstrated that healthy rats exhibited avoidance of a sick social partner (previously injected with LPS) as indexed by several measures of social behavior. Indeed, keeping a distance from sick conspecifics may provide the adaptive benefit of preventing the spread of infectious diseases [47]. Similar pathogen avoidance responses have been reported in male rats toward IL-1 β treated females [48], foreign antigen injected females [49], LPS injected males [50], and tumor-bearing conspecifics [51]. Thus, these data concur with previous reports of illness-induced social avoidance and demonstrate that this avoidance response is observable using this modified social interaction test [28, 34]. This fits well with the concept of a “behavioral immune system” [26, 52] in which behavioral strategies for preventing spread of infection may be as critical to survival as actual host defense (immunological) responses.

While the mechanism responsible for social avoidance of a sick conspecific was not specifically studied here, it seems likely that odor cues emitted by a sick social partner are a primary driving factor in this response. Acute illness is associated with the release of aversive odor cues from sick animals. Importantly, the release of sickness-related odor cues is a downstream consequence of inflammatory processes, including cytokine signaling and other aspects of immunological function, within the sick subject (see [53] for a recent review). These odor cues provide critical information regarding health status, which is then utilized by conspecifics to guide social investigation toward (approach) or away (avoidance) from the odor donor [53, 54]. Sickness-related odor cues in rodent species are released over protracted periods of time (e.g. over 24 h in the mouse [55]) and are readily transferred to objects that have come in contact with the sick subject, such as bedding. Indeed, naïve rats exposed to bedding collected from LPS-injected rats exhibit a robust avoidance as evidenced by decreased sniffing, behavioral avoidance responses, and increased burying behavior [50].

The majority of the behavioral measures used to index social interaction in the present experiment demonstrated that a short-term history of social isolation principally increased social contact behavior, whereas effects on other social measures (sniffing, approach toward) showed much more modest effects. Importantly, social contact behavior was increased in isolated experimental subjects only when interacting with healthy social partners, or perhaps conversely, that this stimulation of social contact by isolate-housing was attenuated when isolated experimental animals interacted with a sick partner. Thus, short-term social deprivation was able to increase social interactions, but only if the social partner was healthy. Hence, social contact behavior appeared to be highly influenced by the health status of the social partner, even overriding short-term increases in social behavior [28].

When brains of healthy experimental rats were analyzed immediately after the social interaction experience, a modest increase in TNF- α exclusively in the olfactory bulb of both single- and pair-housed experimental rats exposed to sick conspecifics was observed (Figure 3). These results provide preliminary evidence for an anticipatory cytokine response in rats exposed to a sick conspecific. As would be expected, this effect was substantially smaller (~75% increase) in magnitude than the increased TNF- α (or other cytokines) observed in response to actual LPS exposure (~500% increase; see Figure 4). Nevertheless, from an evolutionary perspective, the ability to detect contagious individuals in order to display an appropriate behavioral (avoidance) response would be highly beneficial. The fact that this effect was observed in the OB (but not other structures) is suggestive that sickness-related odor cues might be initially transduced within circuits relevant to the processing of social cues. Further, upon encountering a sick individual, it would be advantageous to mount an immune response when a possible contagion is encountered. Such priming effects have been observed in other studies, such as with the demonstration that stress can lead to priming of subsequent cytokine responses [25, 56, 57], enhanced clearance of bacteria [58], enhanced delayed type hypersensitivity [59], and priming of microglia in brain [60]. Future studies in which the duration of exposure to a sick partner is manipulated might provide additional insight into the nature and kinetics of immunological priming observed here.

A unique aspect of this study was that the manipulation of the social partner animals provided an opportunity to confirm LPS-induced illness by measuring plasma and central cytokines, as well as to determine whether interaction with a normal (pair-housed) or socially deprived (isolate-housed) experimental animal might influence the cytokine response to LPS administration. The effects of administration of an immunogen, such as LPS, on social behavior (see [5] for review) and central cytokines [43] have been extensively studied. Both peripheral and central injection of LPS lead to profound activation of immune pathways, with cytokines such as IL-1 β , TNF- α and IL-6 being robustly increased in the brain [43]. Not surprisingly, and consistent with previous experiments, significant increases in expression of central cytokines were exhibited in social partners injected with LPS (e.g., IL-1 β , TNF- α , IL-6, and I κ B α) across all 4 brain regions examined (i.e., olfactory bulb, PVN, amygdala, and hippocampus).

Interestingly, when LPS injected social partners interacted with isolate-housed experimental rats, significant exacerbation of LPS-induced increases in hippocampal IL-1 β and I κ B α expression were observed relative to sick partners that experienced interaction with a pair-housed experimental subject. These results would suggest that the hippocampus may be uniquely sensitive to the modulatory influence of social deprivation on LPS-induced cytokine expression. Although the reasons for this are presently unclear, the interaction between social processes and cytokines are an intense area of inquiry (see review [6]) and are likely to be the subject of future studies.

Contrary to what was observed on central cytokine expression, plasma cytokines tended to be (nonsignificantly) lower in LPS injected partners, with the exception that plasma IL-6 was elevated. Given that plasma was collected 3.5 h after LPS administration, this pattern of changes seems consistent with the notion that plasma cytokine responses evoked by LPS tend to resolve rather quickly, and that the half-life of cytokines in plasma is relatively short.

Indeed, the only cytokine increased in plasma was IL-6, which is commensurate with the role of IL-6 as a late-responding cytokine with mixed actions [61]. Additionally, it is notable that these measurements were collected after a 30 min social interaction session, which could have impacted plasma cytokine levels as well. Finally, it is worth noting the circulating cytokines were assessed at the protein level using relatively new technology - the BioRad magpix system - which may yield slightly different normative values for plasma cytokines under basal and LPS-stimulated conditions. Nevertheless, these peripheral cytokine data provide important contrast to the large and sustained increase in expression of cytokines in the CNS, suggesting that central (but not circulating) cytokines are predictive of LPS-induced disruptions in social behavior. No other changes in central cytokine expression were impacted by partners' housing condition in LPS-treated subjects. Overall, our results support the concept that the immediate social environment following an immune challenge can influence the early innate immune response to pathogens, which culminate in sickness behavior.

In the present study, the avoidance response exhibited by healthy experimental subjects toward sick social partners was not correlated with plasma CORT concentrations. Corticosterone is a potent anti-inflammatory steroid hormone [62, 63]. As such, it is possible that CORT *per se* may mediate the aversive properties of urinary odor, which may have been a mechanism by which sick partners induce social avoidance in healthy rats. Prior studies, however, have shown that adrenalectomy did not influence the odor properties of rats [64]. Furthermore, this is consistent with previous work showing the avoidance response of healthy rats to illness-related odors was not correlated with changes in plasma CORT, or testosterone concentrations from odor-donor animals injected with LPS, thus suggesting that the inflammatory cascade is likely to mediate aversive properties of odor that function to signal illness state to conspecifics [17]. Additionally, it is important to consider that CORT (Table 1) and plasma cytokine concentrations (Table 2) in this experiment were collected from behaviorally tested animals 30 min after the test session, which corresponds to 3.5 h after LPS injection. Thus, comparisons between LPS- and saline- social partners do not represent HPA-axis reactivity purely to LPS; rather the reported values reflect the interaction between LPS exposure and the social behavior testing procedure. The only effect that emerged in plasma CORT was a modest potentiation of CORT release in LPS-injected rats, which was largely to be expected. In sum, we have included these data to provide initial evidence that corticosterone is not likely to be a contributing factor in the behavioral or cytokine effects reported here.

One possible underlying explanation for the avoidance behavior is that the subjects are experiencing visceral disgust in response to sickness cues [65]. The insular cortex has been identified as a key structure in the processing of social disgust, and the behavioral avoidance may be reflected in changes in serotonergic input to this region [66]. Although the preponderance of evidence suggest that illness-associated odor cues are a primary means of illness-related communication in rodents (see [53]), we cannot rule out the possibility that other cues emitted by the sick conspecific (vocalizations, change in ambient temperature or movement, or other visual cues) could be important for communication of illness-related information. Furthermore, it is possible that the avoidance response observed in healthy partners exposed to sick conspecifics could reflect transient exposure to LPS on the fur or in

fecal material from LPS-injected rats. Another question that arises is why the healthy subjects exposed to LPS-injected partners had an increase in TNF- α but not other pro-inflammatory cytokines. TNF- α is typically the first major pro-inflammatory cytokine to increase in response to LPS challenge [61], so it is possible that IL-1 and IL-6 would show a similar increase in future studies if gene expression levels were measured at later timepoints.

Overall, the results from this large, comprehensive study allow for several preliminary conclusions. First, the ability of brief social deprivation to increase the expression of social behavior was observed only when isolated rats were tested against a healthy social partner, suggesting that the risk of interacting with a sick partner outweighs the reward/benefit of a brief social interaction. Second, a modest but reliable increase in TNF- α exclusively within the olfactory bulb provides preliminary evidence suggesting that exposure to a sick partner might elicit an anticipatory neuroimmune response in healthy conspecifics. And finally, isolate-housing was associated with a potentiated cytokine response that was observed uniquely in the hippocampus (but not other structures or in blood), which suggests the hippocampus may exhibit significant plasticity in the neuroimmune response as a result of social experience. Together, these findings may have important implications for how social experience contributes to positive health, and conversely, how adverse social experiences (i.e., social deprivation) may exacerbate health conditions across the lifespan.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by NIH grant number R01AG043467 to T.D., the Center for Development and Behavioral Neuroscience at Binghamton University, and São Paulo Research Foundation FAPESP (2012/03372-3; 2013/25146-8) to E.K.H.. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the above stated funding agencies. The authors have no conflicts of interest to declare.

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Highlights

- Isolation increased social behavior toward healthy but not sick social partners.
- Interaction with a sick social partner modestly increased TNF- α in olfactory bulb.
- Social isolation potentiated IL-1 β and IL-6 responses to LPS only in hippocampus.
- In social partners, central cytokine expression was predictive of sickness behavior.
- Social experience may be an important contributor to health across the lifespan.

Figure 1. (A) Experimental design, hypotheses, and brain punch locations. (A) Healthy subjects were pair or isolate housed for 5 days prior to social interaction with a social partner that received either an LPS or saline injected 3 hours prior to the beginning of the social test. Immediately after testing, brains were collected and sliced on a cryostat, and bilateral tissue punches (1.2 – 2.0 × 1.0 – 2.0 mm) were then taken from structures of interest; the olfactory bulb (B), paraventricular nucleus of the hypothalamus (PVN; C), amygdala (D), and hippocampus (E).

Figure 2. Social behaviors exhibited by housing-manipulated subjects (isolated or paired housed) in 30 min of exposure to partner animals which were either given saline or LPS (250 μ g/kg, i.p.) 3 h prior to the test session. (A) frequency of sniffing, (B) frequency of social contact, (C) frequency of movement away from partner, (D) frequency of movement toward partner. Data are expressed as mean $\pm$ S.E.M. * indicates a main effect of LPS injection. Different letters across groups represent significant differences.

Figure 3.

RT-PCR results from healthy pair or isolate housed rats that interacted with healthy or sick partners. The relative expression TNF- α is shown for the olfactory bulb are presented here. For a full panel of structures and gene expression targets, see Supplementary Figure 1. Data are expressed as mean $\pm$ S.E.M. ^ indicates a main effect of the social partner's LPS injection.

Figure 4. RT-PCR results from rats that were injected with either LPS or saline and interacted with healthy pair or isolate housed conspecifics. Relative expression levels of IL-1 β , IL-6, TNF- α , I κ B α , and c-Fos are shown for the olfactory bulb (A-E), PVN (F-J), amygdala (K-O), and hippocampus (P-T). Data are expressed as mean $\pm$ S.E.M. * indicates a main effect of LPS injection, and # indicates a main effect of partner's housing condition. Where significant interactions were found, different letters are used to denote difference between groups

Table 1

Plasma corticosterone concentrations of housing -manipulated and LPS-injected subjects post-social testing.

Groups	Corticosterone (µg/dl)
<i>Experimental test subjects</i>	
Pair housed – Saline-injected partner	27.60 ± 2.43
Pair housed – LPS-injected partner	27.88 ± 2.33
Isolated housed – Saline-injected partner	25.37 ± 1.25
Isolated housed – LPS-injected partner	25.44 ± 3.60
<i>Social Partners (healthy or sick)</i>	
Saline-injected – Pair housed partner	39.48 ± 2.97
LPS-injected – Pair housed partner	44.08 ± 4.00
Saline-injected – Isolated housed partner	39.15 ± 4.74
LPS-injected – Isolated housed partner	43.68 ± 1.90

Data are expressed as mean ± S.E.M.

Table 2

Injection	Saline		LPS	
	Partner status	Pair	Isolate	Pair
IL-1 α		1.90 $\pm$ 0.10	1.79 $\pm$ 0.20	1.43 $\pm$ 0.26
IL-1 β		22.21 $\pm$ 1.31	22.35 $\pm$ 2.99	16.45 $\pm$ 3.32
IL-2		2.50 $\pm$ 0.12	2.30 $\pm$ 0.24	1.90 $\pm$ 0.31
IL-4		1.05 $\pm$ 0.06	1.03 $\pm$ 0.12	0.79 $\pm$ 0.15
IL-5		1.88 $\pm$ 0.10	1.75 $\pm$ 0.17	1.65 $\pm$ 0.15
IL-6		2.69 $\pm$ 0.59	2.91 $\pm$ 0.81	4.73 $\pm$ 1.22
IL-10		4.70 $\pm$ 0.27	4.28 $\pm$ 0.39	4.28 $\pm$ 0.40
IL-12p70		2.29 $\pm$ 0.14	2.22 $\pm$ 0.28	1.64 $\pm$ 0.32
IL-13		1.66 $\pm$ 0.09	1.66 $\pm$ 0.20	1.25 $\pm$ 0.24
GM-CSF		0.69 $\pm$ 0.07	0.72 $\pm$ 0.10	0.54 $\pm$ 0.10
INF- γ		3.01 $\pm$ 0.16	2.79 $\pm$ 0.34	2.57 $\pm$ 0.49
TNF- α		1.21 $\pm$ 0.14	1.24 $\pm$ 0.14	1.24 $\pm$ 0.23
				1.30 $\pm$ 0.16

Plasma cytokine concentrations (pg/100 μ g total protein) of saline or LPS-injected rats 3.5 hours after I.P. administration of either saline or LPS (250 μ g/kg). Data are expressed as mean $\pm$ S.E.M., and bold indicates a main effect of LPS injection.