Behavioral/Systems/Cognitive

Neonatal Infection-Induced Memory Impairment after Lipopolysaccharide in Adulthood Is Prevented via Caspase-1 Inhibition

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We have reported that neonatal infection leads to memory impairment after an immune challenge in adulthood. Here we explored whether events occurring as a result of early infection alter the response to a subsequent immune challenge in adult rats, which may then impair memory. In experiment 1, peripheral infection with *Escherichia coli* on postnatal day 4 increased cytokines and corticosterone in the periphery, and cytokine and microglial cell marker gene expression in the hippocampus of neonate pups. Next, rats treated neonatally with *E. coli* or PBS were injected in adulthood with lipopolysaccharide (LPS) or saline and killed 1–24 h later. Microglial cell marker mRNA was elevated in hippocampus in saline controls infected as neonates. Furthermore, LPS induced a greater increase in glial cell marker mRNA in hippocampus of neonatally infected rats, and this increase remained elevated at 24 h versus controls. After LPS, neonatally infected rats exhibited faster increases in interleukin-1β (IL-1β) within the hippocampus and cortex and a prolonged response within the cortex. There were no group differences in peripheral cytokines or corticosterone. In experiment 2, rats treated neonatally with *E. coli* or PBS received as adults either saline or a centrally administered caspase-1 inhibitor, which specifically prevents the synthesis of IL-1β, 1 h before a learning event and subsequent LPS challenge. Caspase-1 inhibition completely prevented LPS-induced memory impairment in neonatally infected rats. These data implicate IL-1β in the set of immune/inflammatory events that occur in the brain as a result of neonatal infection, which likely contribute to cognitive alterations in adulthood.

Key words: **cytokines; interleukin-1; postnatal; immune; cognition; microglia**

Introduction

Early exposure to infectious agents may have significant consequences for the development and function of physiological systems throughout an individual's lifespan, a phenomenon termed "perinatal programming" (Barker et al., 1995). For instance, neonatal exposure to bacterial products [lipopolysaccharide (LPS)] in rats influences reactivity to stress, immune regulation, and susceptibility to disease in adulthood (Shanks et al., 2000; Hodgson et al., 2001; Boisse et al., 2004). Furthermore, an increasing body of evidence from both animal (Hornig et al., 1999; Shi et al., 2003) and human (Rantakallio et al., 1997; Nelson and Willoughby, 2000) studies suggests that perinatal events involving the immune system may contribute to the development of behavioral or neuropsychiatric disorders, including schizophrenia, autism, and cerebral palsy.

There is strong evidence in rodents that proinflammatory cytokines [e.g., interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα)] generated by the maternal or fetal immune system during perinatal infection are important mediators of abnormal brain development and increased risk for neurodevelopmental disorders (Cai et al., 2000; Urakubo et al., 2001; Pang et al., 2003; Richardson-Burns and Tyler, 2004; Yu et al., 2004). For example, perinatal endotoxin administration induces cytokine expression, apoptosis, and glial activation in the immature rat brain, factors that have been linked to white matter injury (Cai et al., 2003; Golan et al., 2005). Behavioral alterations in particular may be the result of damage by cytokines during development to the cortex and especially the hippocampus, a brain region important to the pathology of behavioral and cognitive disorders (Bauman et al., 1997). Cytokine receptors are distributed throughout the brain, with high densities in the hippocampus (Cunningham and De Souza, 1993). For this reason, the hippocampus is thought to be particularly vulnerable to immune-related alterations (Lynch et al., 2004).

We have reported that neonatal exposure to bacteria (*Escherichia coli*) in rats is associated with memory impairment in adulthood (Bilbo et al., 2005). Remarkably, however, this impairment is only observed if an immune challenge (LPS) is administered immediately after the learning experience. These data are, to our knowledge, unique within the perinatal infection literature, because behavioral impairments are only observed in neonatally infected rats after a subsequent immune challenge in adulthood. Evidence from animal models suggests that cytokine actions within the brain, in particular IL-1β, may influence cog-

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nitive processes (Banks et al., 2002). Here we tested the hypothesis that events occurring as a result of early infection alter the brain cytokine response to a subsequent immune challenge in adult rats, which in turn influences the processes that store memory.

We demonstrate that, in neonate pups, peripheral *E. coli* infection increases cytokines and corticosterone in the periphery and induces IL-1β and microglial cell marker gene expression in the hippocampus. Furthermore, adult rats infected as neonates exhibit increased basal and LPS-induced gene expression of glial cell markers within the hippocampus and exaggerated IL-1β responses to LPS within the hippocampus and adjacent parietal cortex compared with controls. LPS impairs memory for a novel context in rats infected as neonates. However, blocking the synthesis of brain IL-1β 1 h before context exploration and subsequent LPS completely prevents the memory impairment in neonatally infected rats.

Materials and Methods

Animals

Adult male and female Sprague Dawley rats (70 d) were obtained from Harlan Sprague Dawley (Indianapolis, IN) and housed in same-sex pairs in polypropylene cages with *ad libitum* access to food and water. The colony was maintained at 22°C on a 12 h light/dark cycle (lights on at 5:00 A.M.). After acclimation to experimental conditions, males and females were paired into breeders. Sentinel animals were housed in the colony room and screened periodically for the presence of common rodent diseases; all screens were negative. All experiments were conducted with protocols approved by the University of Colorado Animal Care and Use Committee.

Neonatal manipulations

Female breeders were visually examined daily for confirmation of pregnancy, and male breeders were removed from cages before the birth of pups [postnatal day 0 (P0)]. Female pups were culled to two per litter on P4. All studies were limited to males. P4 was initially chosen based on a model of neonatal LPS challenge in rats (Shanks et al., 2000; Hodgson et al., 2001) and represents a time relatively comparable with the third trimester in humans during which significant brain growth occurs (Dobbing and Sands, 1979; Rodier, 1980).

Bacterial culture

E. coli culture (ATCC 15746; American Type Culture Collection, Manassas, VA) vial contents were hydrated and grown overnight in 30 ml of brain-heart infusion (BHI) (Difco, Detroit, MI) at 37°C. Cultures were aliquoted into 1 ml stock vials supplemented with 10% glycerol and frozen at −20°C. One day before injections, a stock culture was thawed and incubated overnight in 40 ml of BHI at 37°C. The number of bacteria in cultures was read using a microplate reader (Bio-Tek Instruments, Winooski, VT) and quantified by extrapolating from previously determined growth curves. Cultures were centrifuged for 15 min at 4000 rpm, the supernatants were discarded, and the bacteria were resuspended in the dose-appropriate volume of sterile Dulbecco's PBS (Invitrogen, Carlsbad, CA).

Bacterial injections

Male pups were injected subcutaneously on P4 with either 1×10^6 colony forming units of live bacterial *E. coli* suspended in 0.1 ml of PBS, or 0.1 ml of PBS. This dose was originally selected because it induced cytokine production in the periphery and brain of pups without being lethal in pilot studies. Noninjected pups have been tested previously, and results did not significantly differ from PBS-injected controls (Bilbo et al., 2005); therefore, noninjected pups were not included in these studies. All pups were removed from the mother at the same time and placed into a clean cage with bedding, injected individually, and returned to the mother as a group. Elapsed time away from the mother was <5 min. All pups from a single litter received the same treatment because of concerns over possible cross-contamination from *E. coli*. All injections were given between 2:00 P.M. and 4:00 P.M. All male pups for adult analyses were weaned on P21 and housed in sibling pairs; remaining female pups were culled. To control for possible litter effects, a maximum of two pups per litter were assigned to a single experimental group.

Experiment 1

Neonatal inflammatory response

We determined the time course of peripheral cytokine and corticosterone production, and hippocampal cytokine and glial cell marker gene expression in neonate pups after peripheral *E. coli* infection. In contrast to models of perinatal immune activation, which use large or repeated doses of cytokines or LPS (Plagemann et al., 1998; Shanks et al., 2000; Hodgson et al., 2001), we used live, replicating *E. coli* to induce cytokine release more comparable with an infection during the neonatal period.

Injections and tissue collection. Pups from a total of eight litters were injected with *E. coli* as described and were killed 2, 8, 24, 48, or 72 h ($n = 8$ per group) later. Pups from a total of four litters were injected with PBS and were killed 2 or 48 h ($n = 8$ per group) later. Brains collected at each time point were distributed across litters. The PBS time points were chosen to control for the possible effect of developmental age on gene expression. After rapid decapitation, trunk blood samples were collected and placed on ice. Blood was centrifuged at 10,000 rpm at 4°C for 10 min, and supernatant was removed and stored in microcentrifuge tubes at −20°C until assayed for cytokine and corticosterone concentrations. Brains were quickly removed from the skull and placed into an ice-cold PBS slurry for 1 min to firm the tissue for dissection. The hippocampus was immediately dissected out on an ice-chilled plate, snap frozen in liquid nitrogen, and stored at −70°C until processing for mRNA. Gene expressions of IL-1β, IL-6, TNFα (proinflammatory), IL-10 (anti-inflammatory), CD11b (microglial cell marker), and GFAP (astrocyte cell marker) were determined using real-time reverse transcription (RT)-PCR. Gene expression of *E. coli* 23S, a portion of ribosomal RNA unique to bacteria (Van Camp et al., 1993), was measured to determine whether live *E. coli* entered the brain in response to peripheral injection.

Adult inflammatory response

We determined whether cytokine production in the periphery and brain, and cytokine and glial cell marker gene expression in the hippocampus, were altered in adult rats as a consequence of early infection. These measures were analyzed basally and in response to a subsequent immune challenge.

LPS injections. Adult rats (2–3 months) treated neonatally with either *E. coli* or PBS were weighed 1 d before receiving 0.1 ml of intraperitoneal injections of 25 μg/kg LPS (serotype 0111:B4, lot 072K4096; Sigma, St. Louis, MO) or an equal volume of sterile saline. LPS-injected rats were anesthetized with sodium pentobarbital (Abbott Laboratories, Chicago, IL) 1.5, 3, 4, 8, or 24 h later ($n = 6$ per group), placed on ice, and transcardially perfused with cold saline for 1 min. Saline-injected rats were similarly perfused 24 h later ($n = 6$ per group). Based on initial results in LPS-injected rats, a second group of rats was injected with LPS and perfused 1 h later ($n = 9$ per group). All injections occurred between 8:30 A.M. and 10:00 A.M., and injection and collection times were counterbalanced across groups to control for circadian changes.

Serum and tissue collection. Blood samples were drawn via cardiac puncture just before perfusion and placed on ice. Blood was centrifuged at 10,000 rpm at 4°C for 10 min, and supernatant was collected and stored at −20°C until assayed for circulating cytokines, endotoxin, and corticosterone concentrations. The pituitary and brain were quickly removed after decapitation. Brains were immediately dissected into prefrontal cortex, hypothalamus, hippocampus, and adjacent posterior cortex on an ice-chilled plate. The hippocampus was then cut in half along the sagittal plane, and half regions to be used for mRNA and protein analysis were placed into separate RNase- and endotoxin-free microcentrifuge tubes and snap frozen in liquid nitrogen. All other regions were placed individually into microcentrifuge tubes and snap frozen. Tissues were stored at −70°C until processing. Hippocampal hemispheres collected for assessment of protein versus mRNA were alternated between animals and distributed equally across groups. Gene expressions of IL-

Table 1. Primer sequences used for amplification of rat cDNAs

Gene	Forward primer 5′-3′
Interleukin-1β	gaagtcaagaccaaagtgg
Interleukin-10	taagggttacttgggttgcc
Interleukin-6	acttcacagaggataccac
Tumor necrosis factor α	cttcaagggacaaggctg
GFAP	agggacaatctcacacagg
CD11b	ctgggagatgtgaatggag
E. coli 23S rRNA	ttggctaatcctggtcggac
Glyceraldehyde-3-phosphate dehydrogenase	gtttgtgatgggtgtgaacc

1β, IL-10, CD11b, and GFAP were determined using real-time RT-PCR. Because significant changes in hippocampal IL-1β and IL-10 gene expression occurred in response to *E. coli* in neonates, gene expression analysis in adult rats was limited to these two cytokines.

Hippocampal gene expression analysis

Total RNA was isolated from hippocampal tissue based on the TRIzol method of Chomczynski and Sacchi (1987) and has been described in detail previously (Frank et al., 2005). Briefly, after RNA isolation and enrichment, samples were DNase-treated (DNA-free kit; Ambion, Austin, TX) to remove contaminating DNA from total nucleic acid and requantitated before cDNA synthesis using the SuperScript II First-Strand Synthesis System for RT-PCR (Invitrogen). PCR amplification of cDNA was performed using the Quantitect SYBR Green PCR kit (Qiagen, Valencia, CA). cDNA (1 μl) was added to a reaction master mix (25 μl) containing 5 mM $MgCl_2$, HotStar *Taq*DNA polymerase, SYBR Green I, dNTPs, fluorescein (10 nM), and gene-specific primers (500 nM each of forward and reverse primer).

Primer specifications. cDNA sequences were obtained from GenBank at the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov). Primer sequences (Table 1) were designed using an online Oligo Analysis & Plotting Tool (Qiagen) and tested for sequence specificity using the basic local alignment search tool at NCBI. Primer specificity (Proligo, Boulder, CO) has been verified previously in our laboratory by melt curve analysis (Johnston et al., 2004).

Quantitative real-time PCR. Samples were run in triplicate. Formation of PCR product was monitored in real time using the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Threshold cycle (C_T) (number of cycles to reach threshold of detection) was determined for each reaction. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Pfaffl, 2001).

Cytokine analysis

IL-1β, IL-6, and TNFα were measured in serum using commercially available ELISA kits (R & D Systems, Minneapolis, MN). Dissected brain regions from adult rats were added to 0.25–1.0 ml of cold Iscove's culture medium containing 5% fetal calf serum and a cocktail enzyme inhibitor (in mM: 100 amino-*n*-caproic acid, 10 EDTA, 5 benzamidine-HCl, and 0.2 phenylmethylsulfonyl fluoride). Total protein was mechanically dissociated from tissue using an ultrasonic cell disruptor (Heat Systems, Farmingdale, NY). Sonicated samples were centrifuged at 14,000 rpm at 4°C for 10 min. Supernatant was removed and stored at 4°C (<24 h) until assayed for IL-1β using an ELISA kit (R & D Systems). To adjust for variability in tissue sample sizes, total protein concentrations were determined by Coomassie Brilliant Blue AG-250 protein assay (Bradford, 1976). Brain cytokine data are expressed as picograms per 100 μg of total protein. All ELISAs were run according to the instructions of the manufacturer.

Radioimmunoassay procedures

Total serum corticosterone concentrations were assessed for neonates and adults in separate assays using an in-house radioimmunoassay procedure as described previously (Bilbo et al., 2005). The detection limit of the assay was 0.5 μg/ml for a 20 μl sample, and the intraassay coefficients of variability for each assay were 8 and 10%, respectively.

Serum endotoxin assessment

Serum levels of endotoxin were determined to confirm accuracy of LPS injections in adult rats using an enzymatic assay, according to the procedure outlined by Bio-Whittaker (Walkersville, MD). The detection limit of the assay is 0.02 U/ml. Serum was diluted 1:20.

Experiment 2

Neonatal exposure to bacteria leads to impaired memory in adulthood if a peripheral immune challenge (LPS) is administered immediately after the learning event (Bilbo et al., 2005). The same dose of LPS has no effect on memory in control rats. The goal of this experiment was to test the hypothesis that neonatal infection-induced alterations in IL-1β production after an immune challenge in adulthood lead to memory impairment. If this is the case, then preventing the synthesis of IL-1β within the brain before the learning event and subsequent LPS should prevent memory impairment in neonatally infected rats. A caspase-1 inhibitor, which specifically prevents the synthesis of IL-1β, was administered centrally to allow a test of the hypothesis that altered production of brain IL-1β in response to a normal immune signal from the periphery underlies the memory impairment.

Behavioral procedures

Memory was tested using a modified version of contextual fear conditioning known as the context preexposure task (Fanselow, 1990; Rudy et al., 2004). This paradigm assesses the rat's memory for a recently explored context. In this task, when normal rats are placed into a conditioning context and immediately receive a footshock, they later display little or no conditioned fear (freezing) to the context. This absence of fear to the context is thought to occur because the immediately shocked rat did not have the opportunity to sample the environment and store a representation of its features. However, if a rat is preexposed to the context the day before, immediate shock conditioning will then produce substantial freezing on a subsequent test day (Fanselow, 1990; Westbrook et al., 1994; Rudy and O'Reilly, 2001). Preexposure should facilitate the amount of conditioning produced by the immediate shock because it allows the rat to have established a memory of the context before the immediate shock; it is this retrieved memory representation of the context that is then associated with the immediate shock (Rudy and O'Reilly, 2001; Rudy et al., 2002).

The preexposure task was chosen for several reasons. First, each stage of the procedure critically depends on the hippocampus, and the hippocampus may be particularly vulnerable to immune-related alterations (Barrientos et al., 2002; Rudy et al., 2002; Matus-Amat et al., 2004). Second, it allows the rats to learn the context incidentally, independent of its association with the aversive shock. Third, learning occurs within a single trial, thus allowing a distinct time period for manipulation (e.g., immune challenge) after the learning experience.

Apparatus. The conditioning context consisted of one of two identical Igloo ice chests (in cm: 54 length × 30 width × 27 height) with white interiors. An activated 24 V direct current DC light bulb (14.14 lux) was mounted on the ceiling of each chest. The conditioning chambers (in cm: 26 length × 21 width × 24 height), placed inside each chest, are made of clear plastic and have window screen tops. The 2 s, 1.5 mA shock was delivered through a removable floor of stainless steel rods (model E63-23-MOD001; Coulbourn Instruments, Allentown, PA). Each rod was 0.5 cm in diameter, spaced 1.75 cm center to center, and was wired to a shock generator and scrambler (Coulbourn Instruments model H13-16). The chamber was cleaned with water before each animal was placed inside.

Caspase-1 inhibitor injections. Adult rats treated neonatally with either *E. coli* or PBS were weighed 1 d before injection with either the specific caspase-1 inhibitor Ac.YVAD-cmk (Ac-Tyr-Val-Ala-Asp-chloromethyl ketone; lot B60162; Calbiochem, Darmstadt, Germany) at a dose of 300 ng/rat in 10 μl or an equal volume of vehicle (0.6% DMSO in sterile saline). Injections were given via the intra-cisterna magna (ICM) under light halothane anesthesia (<2 min) using a 30 gauge needle connected by polyethylene-10 tubing to a 50 μl Hamilton syringe. No incision was made, and thus the complications of surgery were avoided (e.g., infection and glial activation). Previous studies using radiolabeled inulin injected

via the ICM have demonstrated a pattern of labeling throughout the brain within 1.5 h (Proescholdt et al., 2000).

Context preexposure. One hour after ICM injections, rats were transported two at a time in a lidded, black ice bucket to the experimental testing room where they were placed into the conditioning context, allowed to freely explore for several minutes, and were then transported back to their home cage, where they remained ~40 s before the next preexposure. This procedure was repeated six times. Animals remained in the novel context for 5 min on the first exposure and for 40 s on the five subsequent exposures. The rats were transported in the black bucket throughout the experiment.

LPS injections. Immediately after the last exposure to the context, half of the rats in each group then received 0.1 ml intraperitoneal injections of 25 μg/kg LPS suspended in sterile saline and were returned to their home cages. The remaining half received no injection to replicate the previous behavioral study (Bilbo et al., 2005).

Immediate shock. Twenty-four hours after context preexposure, each rat was taken individually from their home cage and transported in the black bucket to the conditioning context. There, they received a single 2 s footshock immediately after being placed in the context. They were quickly removed from the chamber and transported back to their home cage.

Testing. Contextual fear was assessed 24 h after immediate shock by placing the rat in the conditioning context for 6 min. Each rat was observed by an observer blind to experimental conditions and judged as either freezing or active every 10 s, at the instant the sample was taken. Freezing represents rats' dominant defensive fear response and is characterized by an immediate suppression of behavior accompanied by immobility, shallow breathing, and a variety of other autonomic changes, including an increase in heart rate and pilo-erection. Freezing in these experiments was defined as the absence of all visible movement, except for respiration. Scoring began 10 s after the animal was placed into the chamber.

Control injections

ICM injections are designed to deliver the drug centrally into the brain and spinal cord, thus blocking the central but not the peripheral synthesis of IL-1β. To confirm the inhibition of IL-1β synthesis within the brain, but not in the periphery, a separate group of rats from each neonatal group received the caspase-1 inhibitor (ICM) and LPS (intraperitoneally) 1.5 h later and were saline perfused 1.5 h thereafter. The blood, hippocampus, and cortex were collected, and IL-1β concentrations were determined using ELISAs.

Data analysis and statistics

All data were determined to be normally distributed using equal variance tests. Data were analyzed using unpaired two-tailed *t* tests or overall ANOVA tests as appropriate. After significant *F* scores, *post hoc* comparisons (Fisher's protected least significant difference) were performed to further distinguish among groups, and all differences were considered statistically significant if $p < 0.05$.

Results

Experiment 1

Peripheral E. coli *infection increases cytokines and corticosterone in neonate pup serum*

We determined the time course of cytokine and corticosterone responses to *E. coli* in the peripheral serum of neonate pups. Each measure was analyzed as a function of time since injection using one-way ANOVAs; PBS controls were included as distinct time points in ANOVAs. There was a significant main effect of time on IL-1β, IL-6, and corticosterone ($F_{(6,49)} > 2.8$; $p = 0.01$ for all) (Fig. 1). *Post hoc* tests revealed that IL-1β was significantly higher at 24 h versus 2, 8, and 72 h and PBS controls ($p < 0.03$ for all) (Fig. 1*a*) and that IL-6 was higher at 8 and 48 h versus 2 and 72 h and PBS controls ($p < 0.05$ for all) (Fig. 1*b*). Corticosterone was higher at 8 h versus 2 h and PBS controls ($p < 0.03$ for all) and at 8, 24, and 48 h compared with 72 h ($p < 0.05$ for all) (Fig. 1*d*). There were no significant differences in TNFα in the serum.

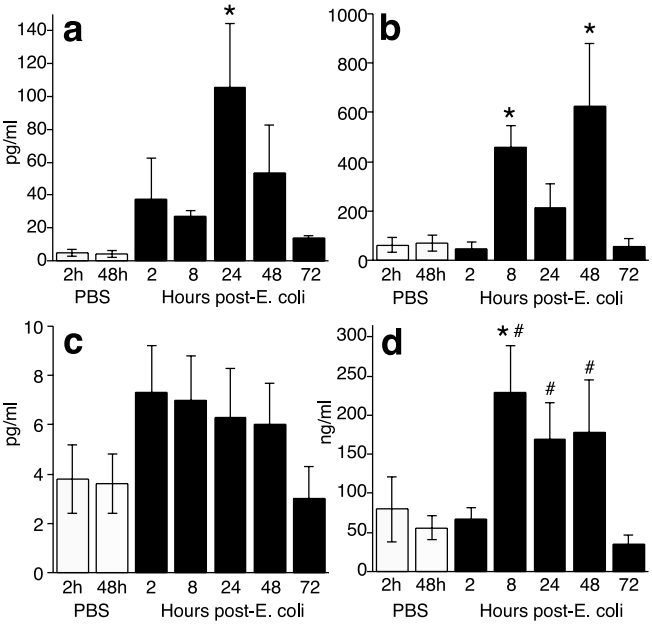

Figure 1. Peripheral *E. coli* infection increases cytokines and corticosterone in neonate pup serum. Pups were injected on P4 with PBS (white bars) or *E. coli* (black bars), and serum was collected at the time points indicated. Data for IL-1β (***a***), IL-6 (***b***), TNFα (***c***) (picomoles per milliliter), and corticosterone (***d***) (nanograms per milliliter) are presented as mean ± SEM. ***a***, IL-1β was increased in response to *E. coli* at 24 h compared with all time points except 48 h; $^{*}p < 0.03$. ***b***, IL-6 was increased in response to *E. coli* at 8 and 48 h compared with all time points except 24 h; $^{*}p < 0.05$. ***d***, Corticosterone was increased in response to *E. coli* at 8, 24, and 48 h; $^{*}p < 0.03$ versus 2 and 72 h and PBS controls; $^{\#}p < 0.05$ versus 72 h.

Peripheral E. coli *infection increases cytokine and microglial cell marker gene expression in the hippocampus of neonate pups*

We determined the time course of hippocampal gene expression after *E. coli* infection in neonate pups. Each gene was analyzed as a function of time since injection using one-way ANOVAs; PBS controls were included as distinct time points in ANOVAs. There was a significant main effect of time on gene expression of IL-1β, IL-10, CD11b, and *E. coli* 23S rRNA ($F_{(6,49)} > 2.0$; $p < 0.05$ for all) (Fig. 2). *Post hoc* tests revealed that IL-1β mRNA was significantly higher at 24 h compared with all other time points ($p < 0.02$ for all) (Fig. 2*a*), and IL-10 mRNA was higher at 48 h compared with every time point ($p < 0.05$ for all) (Fig. 2*b*). CD11b mRNA was higher at 24 and 72 h compared with 8 h and PBS controls ($p < 0.05$ for all) (Fig. 2*e*). *E. coli* 23S rRNA expression was increased at 2 h compared with all other time points ($p < 0.004$ for all) (Fig. 2*g*). There were no significant differences in gene expression of IL-6, TNFα, or GFAP.

Neonatal infection alters gene expression of immune cell markers in adulthood

We determined whether adult hippocampal gene expression for glial cell and cytokine markers differed, at baseline and after LPS, as a consequence of early infection. Each gene was analyzed between neonatal groups as a function of time using two-way ANOVAs; saline controls were included as distinct time points in ANOVAs. There was a significant main effect of time on gene expression of IL-1β, IL-10, CD11b (microglia), and GFAP (astrocytes) ($F_{(6,75)} > 4$; $p < 0.001$ for all) (Fig. 3). These effects were attributable to increases in gene expression in a timewise manner in response to LPS in both neonatal groups. There was a signifi-

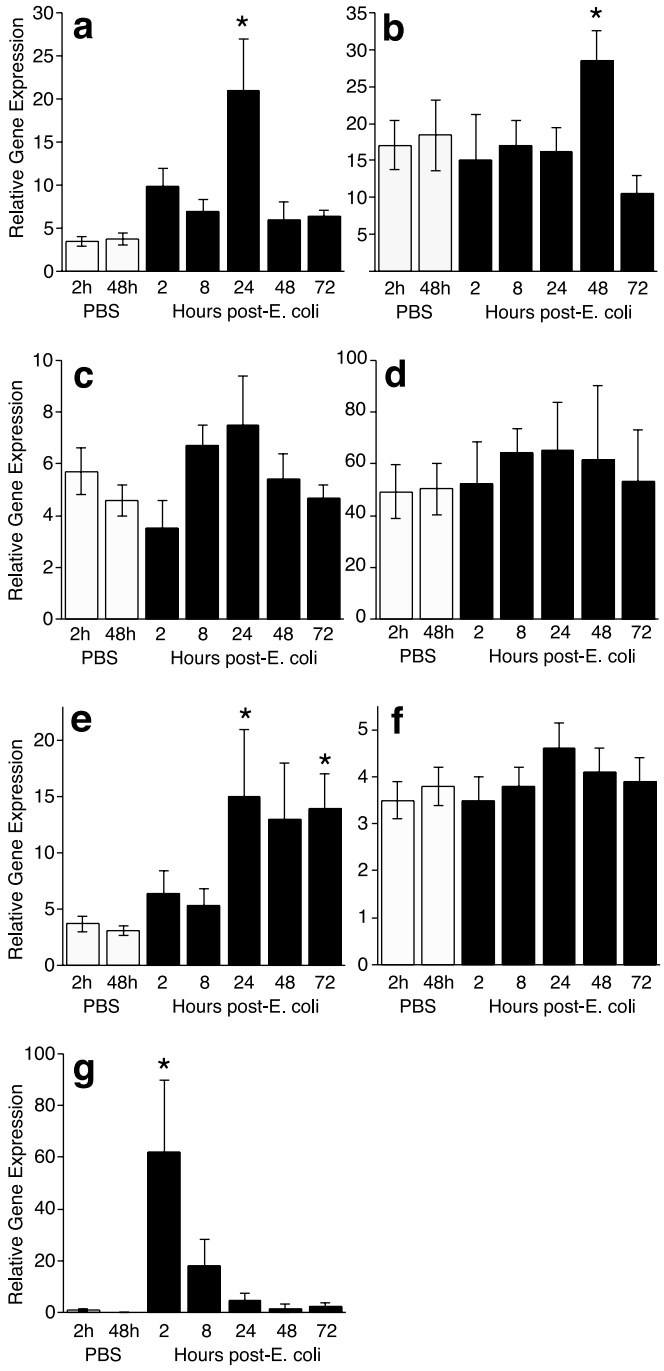

Figure 2. Peripheral *E. coli* infection increases cytokine and microglial cell marker gene expression in the hippocampus of neonate pups. Pups were injected on P4 with PBS (white bars) or *E. coli* (black bars), and brains were collected at the time points indicated. Data for IL-1β (***a***), IL-10 (***b***), IL-6 (***c***), TNFα (***d***), CD11b (***e***; microglial cell marker), GFAP (***f***; astrocyte cell marker), and *E. coli* 23S (***g***; ribosomal RNA) are presented as mean ± SEM relative expression to a housekeeping gene [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)]. ***a***, IL-1β mRNA was higher at 24 h than all other time points. ***b***, IL-10 mRNA was higher at 48 h than all other time points. ***e***, CD11b expression was higher at 24 and 72 h compared with 8 h and PBS controls. ***g***, *E. coli* 23S rRNA expression was higher at 2 h than all other time points; *$p < 0.05$ for all.

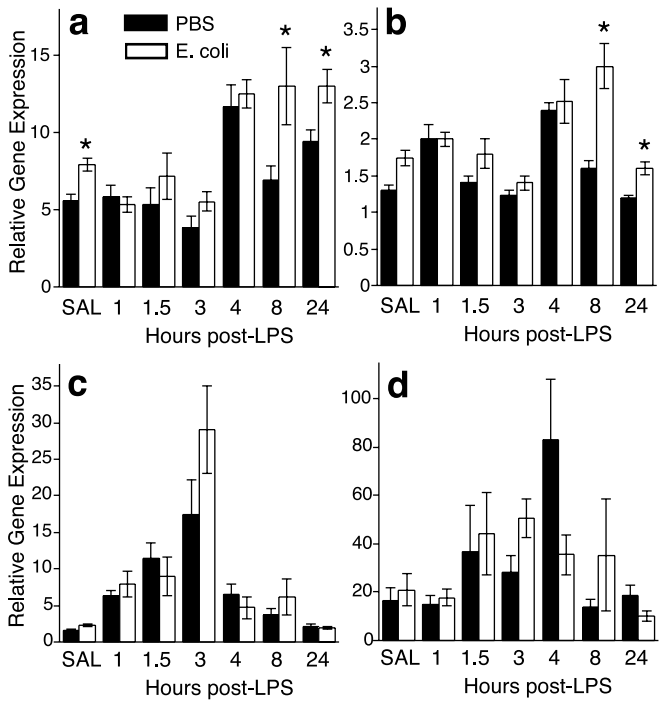

Figure 3. Neonatal infection increases hippocampal gene expression of glial cell markers in adulthood. Adult rats treated on P4 with PBS (black bars) or *E. coli* (white bars) were injected with saline (SAL) or LPS as adults, and brains were collected 1–24 h later. Data for CD11b (***a***), GFAP (***b***), IL-1β (***c***), and IL-10 (***d***) mRNA are presented as mean ± SEM relative expression to a housekeeping gene (GAPDH). ***a***, CD11b expression was higher overall and specifically at baseline (24 h after saline) and 8 and 24 h after LPS in rats infected with *E. coli* as neonates compared with controls. ***b***, GFAP expression was higher overall and specifically 8 and 24 h after LPS in rats infected with *E. coli* as neonates; *$p < 0.05$ for all.

cant main effect of neonatal group on CD11b ($F_{(1,75)} = 13.7$; $p = 0.0004$) (Fig. 3*a*) and GFAP ($F_{(1,75)} = 12.6$; $p = 0.0007$) (Fig. 3*b*), with overall mRNA higher in neonatally infected rats compared with controls. There were also significant group × time interactions on CD11b ($F_{(6,75)} = 2.0$; $p = 0.04$) and GFAP ($F_{(6,75)} = 3.2$; $p = 0.009$). *Post hoc* tests revealed that mRNA was significantly higher at 8 h ($p = 0.04$) and 24 h ($p = 0.03$) post-LPS for CD11b and at 8 h and 24 h ($p = 0.006$ for both) for GFAP in rats infected neonatally with *E. coli*. CD11b mRNA was also higher at baseline (24 h after saline) in neonatally infected rats compared with controls ($p = 0.01$). There were no significant group or interaction effects in IL-1β or IL-10 mRNA.

Neonatal infection alters brain IL-1β production in adulthood

We determined whether brain IL-1β protein production in adulthood differed basally or after LPS as a consequence of early infection. Concentrations were analyzed between neonatal groups as a function of time for each dissected brain region using two-way ANOVAs; saline controls were included as distinct time points in ANOVAs. There was a significant main effect of time on IL-1β in each brain region analyzed (hippocampus, adjacent parietal cortex, prefrontal cortex, hypothalamus, and pituitary; $F_{(6,75)} > 4$; $p < 0.01$ for all) (Fig. 4). These effects were attributable to increases in IL-1β concentrations in a timewise manner in response to LPS in both neonatal groups. Concentrations were extremely low or nondetectable in all regions after saline in both groups. There was a significant main effect of group on IL-1β in parietal cortex ($F_{(1,75)} = 5.7$; $p = 0.01$), with overall concentrations higher in neonatally infected rats compared with controls. There were no significant main effects of group in any other region. However, there were significant group × time interactions in IL-1β concentrations within the hippocampus ($F_{(6,75)} = 2.3$; $p = 0.04$) (Fig. 4*a*) and parietal cortex ($F_{(6,75)} = 3.1$; $p = 0.008$) (Fig. 4*b*). *Post hoc* tests revealed that IL-1β concentrations were significantly higher at 1 h ($p = 0.03$) and 1.5 h ($p = 0.04$) in the hippocampus and at 1 h ($p < 0.0001$), 4 h ($p = 0.01$), and 8 h

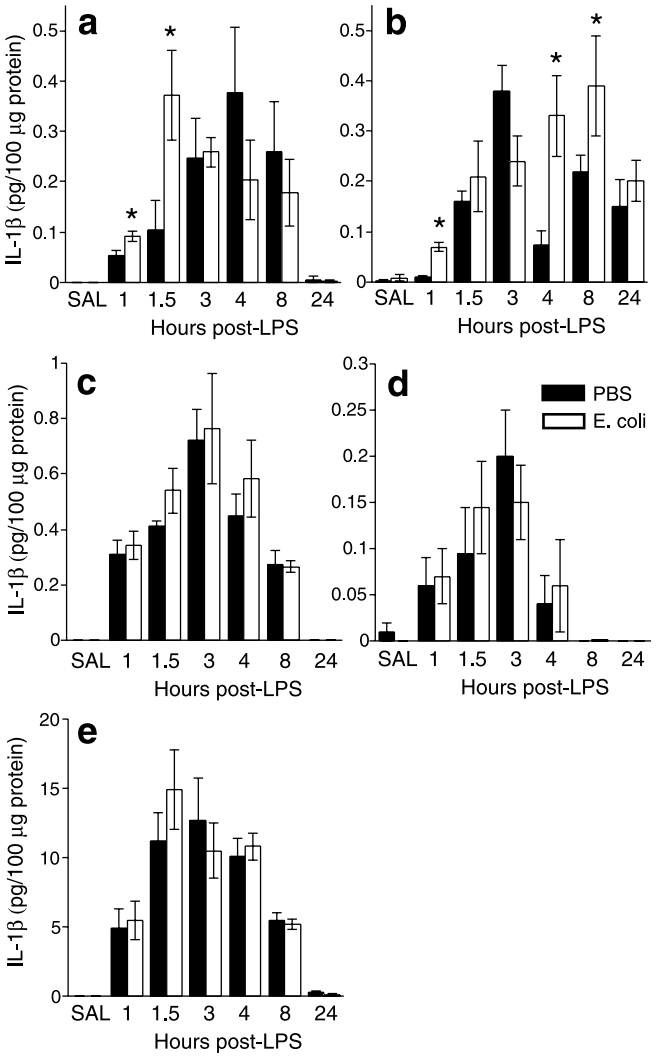

Figure 4. Neonatal infection leads to exaggerated IL-1β responses to LPS in the brains of adult rats. Adult rats treated on P4 with PBS (black bars) or *E. coli* (white bars) were injected with saline (SAL) or LPS as adults, and brains were collected 1–24 h later. Data for hippocampus (***a***), parietal cortex (***b***), prefrontal cortex (***c***), hypothalamus (***d***), and pituitary (***e***) are presented as mean ± SEM picograms per 100 μg of protein. ***a***, IL-1β was higher in hippocampus 1 and 1.5 h after LPS in rats infected with *E. coli* as neonates compared with controls. ***b***, IL-1β was higher in parietal cortex 1, 4, and 8 h after LPS in rats infected with *E. coli* as neonates; *$p < 0.05$ for all. There were no significant differences in other brain regions.

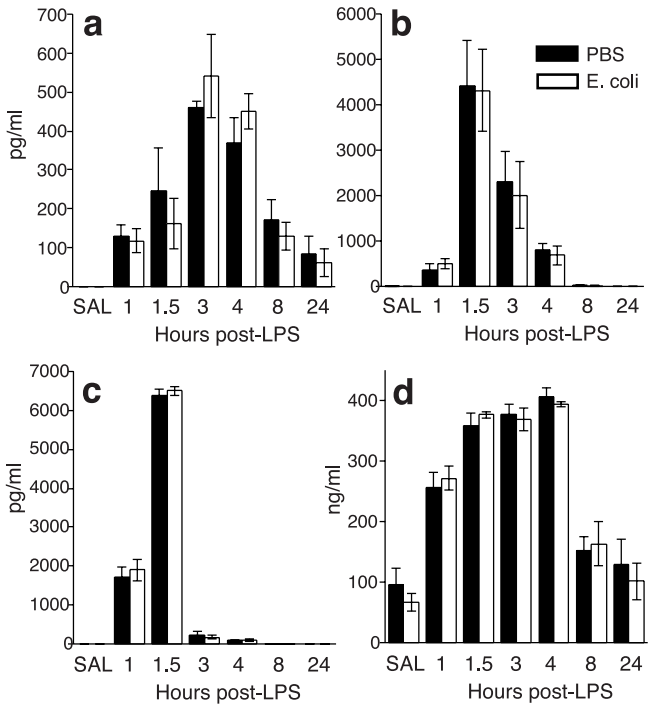

Figure 5. Neonatal infection does not alter peripheral cytokines or corticosterone in adulthood. Adult rats treated on P4 with PBS (black bars) or *E. coli* (white bars) were injected with saline (SAL) or LPS as adults, and serum was collected 1–24 h later. Data for IL-1β (***a***), IL-6 (***b***), TNFα (***c***) (picograms per milliliter), and corticosterone (***d***) (nanograms per milliliter) are presented as mean ± SEM. There were no significant differences between neonatal groups.

($p = 0.04$) in cortex, in rats infected neonatally with *E. coli*. There were no significant interactions in prefrontal cortex, hypothalamus, or pituitary.

Neonatal infection does not alter peripheral cytokines, corticosterone, or body mass in adulthood

The concentrations of serum IL-1β, IL-6, TNFα, and corticosterone were determined for each time point. Injections were verified in LPS-treated rats using endotoxin analysis; one *E. coli*-treated rat from the 1 h group had undetectable endotoxin in the serum and was therefore removed from all other analyses. Two-way ANOVAs (group × time) revealed a significant main effect of time on each cytokine (IL-1β, IL-6, and TNFα) and corticosterone ($F_{(6,75)} > 22$; $p < 0.0001$ for all) (Fig. 5). These effects were attributable to increases in IL-1β concentrations in a time-wise manner in response to LPS in both neonatal groups. However, there were no main effects of group or significant interactions in any measure. To determine whether neonatal infection led to altered body mass in adult rats, body mass was analyzed between rats treated neonatally with either *E. coli* or PBS 1 d before any subsequent manipulation using a two-tailed *t* test. There was no significant difference (PBS, 338 ± 4.8 g, $n = 51$; *E. coli*, 337 ± 6.3 g, $n = 50$; data not shown). We have reported previously that growth rates do not differ beyond weaning between PBS and *E. coli*-treated pups (Bilbo et al., 2005).

Experiment 2

Neonatal infection leads to memory impairment after an LPS challenge in adulthood, and this impairment is prevented via caspase-1 inhibition

Percentage freezing scores in the preexposure task were analyzed between neonatal groups as a function of drug and treatment condition using a three-way ANOVA. There were significant main effects of group ($F_{(1,40)} = 4.5$; $p = 0.04$) and drug ($F_{(1,40)} = 4.7$; $p = 0.03$), as well as significant interactions between group and drug and between group and treatment ($p < 0.03$ for both). Figure 6*A* shows the results for animals that received vehicle 1 h before preexposure. *Post hoc* analysis revealed that rats injected with LPS immediately after the last context exposure displayed marked reduced freezing if they had been infected as neonates ($p = 0.001$). The same dose of LPS had no effect in control rats. Figure 6*B* shows the results for animals that received the IL-1β synthesis inhibitor 1 h before preexposure. There were no differences among groups, because IL-1β synthesis inhibition completely prevented LPS-induced memory impairment in neonatally infected rats.

Caspase-1 inhibition prevented the synthesis of IL-1β in the brain but not in the periphery

IL-1β concentrations in the serum of rats that received the caspase-1 inhibitor and subsequent LPS injections were statisti-

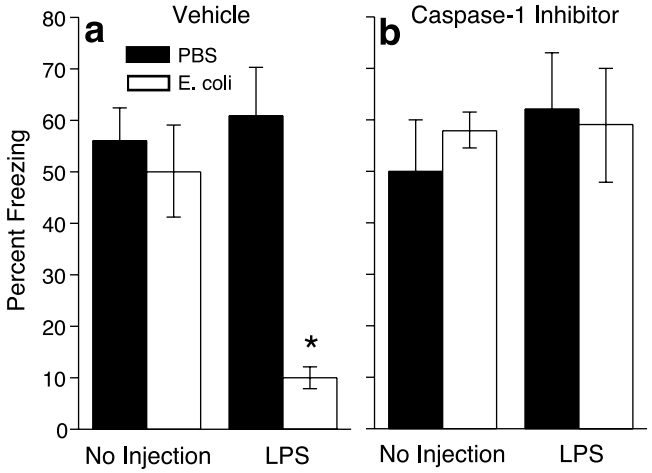

Figure 6. Neonatal infection leads to memory impairment after an LPS challenge in adulthood, and this impairment is prevented via caspase-1 inhibition. Adult rats treated on P4 with PBS (black bars) or *E. coli* (white bars) received either vehicle or a centrally administered caspase-1 inhibitor, which specifically prevents the synthesis of IL-1β, 1 h before context exploration and subsequent LPS challenge. ***a***, Vehicle-treated rats injected with LPS immediately after the last context exposure displayed marked reduced freezing if they had been infected with *E. coli* as neonates; $*p = 0.001$. ***b***, Caspase-1 inhibition before context exposure and subsequent LPS prevented memory impairment in neonatally infected rats.

cally comparable with serum concentrations in LPS-injected adult rats from experiment 1 at 1.5 h (Fig. 5) ($F_{(1,20)} = 1.2$; $p = 0.9$) (PBS, 202 ± 42 pg/ml, $n = 6$; *E. coli*, 283 ± 47 pg/ml, $n = 6$; data not shown). In contrast to serum, the concentrations of IL-1β in the hippocampus and cortex of rats that received the caspase-1 inhibitor and subsequent LPS injection were undetectable. As in experiment 1, adult body mass did not differ between rats treated neonatally with either *E. coli* or PBS (PBS, 383 ± 8.4 g, $n = 24$; *E. coli*, 402 ± 6.9 g, $n = 24$; data not shown).

Discussion

We explored the hypothesis that neonatal infection leads to altered brain cytokine responses after LPS in adulthood, which in turn may interfere with memory. Peripheral *E. coli* injections increased proinflammatory cytokines and corticosterone in the serum, and IL-1β, IL-10, and CD11b gene expression in the hippocampus of neonate pups. In adult rats, basal CD11b expression was elevated in the hippocampus by neonatal infection. Furthermore, LPS induced a significantly greater increase in hippocampal CD11b and GFAP mRNA in adult rats infected as neonates, and this increase remained elevated at 24 h compared with controls. Adults infected as neonates also exhibited faster LPS-induced increases in IL-1β protein within the hippocampus and parietal cortex and a prolonged IL-1β response within the cortex compared with controls. There were no group differences in peripheral cytokines or corticosterone after LPS or 24 h after saline. Finally, caspase-1 inhibition completely prevented LPS-induced memory impairment in adult rats infected as neonates.

Alterations in hippocampal gene expression

Cytokines are increasingly implicated for their role in abnormal brain development in response to perinatal infection (Cai et al., 2000; Urakubo et al., 2001; Pang et al., 2003; Richardson-Burns and Tyler, 2004; Yu et al., 2004). Importantly, the association between infection and developmental disorders involves a number of bacterial and viral agents rather than a specific infection, suggesting a common mechanism such as cytokine release (Fidel et al., 1994; Urakubo et al., 2001; Shi et al., 2003). *E. coli* does appear to enter the neonatal brain after peripheral injection, as measured by increased *E. coli* ribosomal RNA in the hippocampus at 2 h. However, this measure returned to baseline by 24 h, indicating the bacteria were cleared or degraded (Fig. 2*g*). An acute increase in hippocampal IL-1β mRNA occurred 24 h after *E. coli* injection, followed by a peak in anti-inflammatory IL-10 at 48 h. In contrast, proinflammatory IL-6 and TNFα mRNA did not change. These data implicate IL-1β as a key mediator of inflammatory events occurring within the developing brain in response to infection. Along with IL-1β, CD11b mRNA increased 24 h after *E. coli* injection and remained elevated at 72 h, the latest time point analyzed. Microglia and astrocytes are resident immunocompetent cells of the CNS and, in addition to providing support to neurons, play a vital role in the brain response to infection as a major source of cytokines (Benveniste, 1997; Streit, 2002). Microglia in particular are extremely sensitive to disturbances within their microenvironment and may become and remain activated chronically in response to injury or infection (Streit et al., 2004). It is not known how long microglial mRNA remains elevated in the hippocampus in response to neonatal *E. coli*. However, the finding that basal CD11b mRNA was elevated in adult rats infected as neonates suggests that glial alterations in response to early infection persist into adulthood, a question that remains to be fully explored.

Alterations in brain IL-1β

In addition to baseline differences, CD11b and GFAP mRNA increases to LPS were significantly greater in adults infected as neonates. Because glial cells are major producers of cytokines, together these data suggest that rats infected as neonates may be "primed" to exhibit exaggerated cytokine responses to a challenge later in life. There were no group differences in brain IL-1β 24 h after saline, and concentrations were essentially undetectable in both groups, strongly suggesting that neonatal infection does not result in a constant or chronic elevation of IL-1β production within the brain of adult rats. These data are consistent with the lack of behavioral impairment before an LPS challenge. However, after LPS, rats infected neonatally exhibited faster and more prolonged increases in IL-1β within the brain compared with controls. Interestingly, we observed a spike in proinflammatory IL-1β mRNA (3 h after LPS) in *E. coli*-treated rats only and a spike in anti-inflammatory IL-10 mRNA (4 h after LPS) in controls only (Fig. 3), although these interactions were not significant ($p = 0.1$ and 0.07, respectively). Nevertheless, together, these data suggest that the brain response to LPS may be shifted or sensitized toward a proinflammatory response as a result of early infection.

Importantly, exaggerated IL-1β responses to LPS in adulthood were specific to the brain, because no group differences were observed in the periphery in any cytokine. Furthermore, we observed no group differences in corticosterone within the serum, a finding consistent with our previous study (Bilbo et al., 2005) but in contrast to several reports that neonatal LPS exposure potentiates hypothalamic-pituitary-adrenal (HPA) activity in adult rodents (Shanks et al., 2000; Hodgson et al., 2001; Nilsson et al., 2002). The phenomenon that HPA activity is altered after neonatal LPS is not unequivocal, however, because some investigators report changes in overall activity (Shanks et al., 2000; Nilsson et al., 2002), some report altered stress-induced activity only (Hodgson et al., 2001; Hodgson and Knott, 2002), and yet others report no change (Granger et al., 1996; Breivik et al., 2002), consistent with our own findings. This variability may

be attributable to a number of factors, including differences in sampling procedure, rat strain, or variable measured. Ultimately, however, it is difficult to make direct comparisons between the current findings and previous data from neonatal LPS protocols, because we administered a replicating strain of *E. coli*. Together, the current data suggest that altered inflammatory responses within the brain in adult rats infected as neonates are not simply a result of altered immune or stress responses in the periphery and subsequent signal to the brain.

Role of IL-1β in memory impairment

Patients with acquired immunodeficiency syndrome (AIDS)-related dementia, cancer, or chronic inflammatory diseases often exhibit elevated levels of IL-1β co-occurring with cognitive impairment (Gallo et al., 1989; Griffin et al., 1989; Stanley et al., 1994; Meyers, 2000). IL-1β is the most consistently induced cytokine in the brain after peripheral immune stimulation and has a high density of receptors in the hippocampus (Maier and Watkins, 1998). Rats injected with pathological doses of IL-1β either intracerebroventricularly or directly into the dorsal hippocampus display memory impairments (Pugh et al., 1999; Barrientos et al., 2002). Hippocampal long-term potentiation (LTP) is thought to play a major role in the formation of many types of memory (Abraham and Williams, 2003), and exogenous IL-1β inhibits LTP within major hippocampal pathways in a dose-dependent manner (Katsuki et al., 1990; Cunningham et al., 1996). Similarly, peripheral LPS increases IL-1β protein within the hippocampus (Nguyen et al., 1998) and interferes with LTP, with this effect depending on hippocampal IL-1β (Vereker et al., 2000). There is also evidence that IL-1β contributes to demyelination and blood–brain barrier breakdown, processes that can disrupt communication among brain regions (Ferrari et al., 2004). We observed altered IL-1β responses within both the hippocampus and associative parietal cortex in adults infected as neonates, suggesting that impaired or altered communication between these two areas may also have contributed to memory impairment.

There was no effect of LPS on memory in control rats, which is consistent with our previous results (Bilbo et al., 2005) and not unexpected given the relatively low dose of LPS used. These data suggest that neonatally infected rats may be more sensitive to the low dose. It is important to note as well that peak IL-1β concentrations did not differ between groups in any brain region. Rather, IL-1β increased more quickly and remained elevated longer in neonatally infected rats. This finding is of particular interest, because memory consolidation is generally considered to occur in two stages: an early short-term memory stage and a later long-term memory stage (Igaz et al., 2002). A prolonged IL-1β response may therefore coincide and thus critically interfere with one or both stages of memory formation. In support of this hypothesis, inhibiting the synthesis of IL-1β before preexposure and subsequent LPS in experiment 2 completely prevented memory impairment in neonatally infected rats.

Caspase-1 inhibition

Caspase-1 (interleukin-1-converting-enzyme) is required to cleave pro-IL-1β into its mature, biologically active form (Fantuzzi and Dinarello, 1999). The inhibitor Ac.YVAD-cmk specifically blocks the synthesis of IL-1β but has minimal effects on other proinflammatory mediators (e.g., IL-6, TNF, and nitric oxide) (Rabuffetti et al., 2000; Krakauer, 2004). After ICM injections, reductions in IL-1β were specific to the CNS, because IL-1β concentrations were reduced in the brain but not in the periphery. Therefore, the role of brain IL-1β is uniquely implicated in our results. Nonetheless, it should be noted that caspase-1 inhibition may involve a number of other mechanisms. For instance, Ac.YVAD-cmk administration induces neuroprotection after cerebral ischemia, an effect that is attributed to decreases in both apoptotic mechanisms and IL-1β production (Rabuffetti et al., 2000). Ac.YVAD-cmk also prevents peroxynitrite-induced neurotoxicity in neuronal cultures, a metabolite of nitric oxide and strong oxidizing agent thought to be involved in sepsis, inflammation, and apoptosis (Zhang and Rosenberg, 2004). Furthermore, pretreatment of rats with Ac.YVAD-cmk reduces death from sepsis after a lethal dose of LPS, independent of serum reductions in IL-1β (Mathiak et al., 2000). These data are difficult to interpret, however, given the massive dose of LPS and suboptimal dose of Ac.YVAD-cmk administered, as suggested by the authors. Finally, caspase-1 is also involved in the processing of proinflammatory IL-18, the actions for which are much less well defined (Martinon and Tschopp, 2004). Thus, it is possible that caspase-1 inhibition prevents memory impairment in neonatally infected rats via mechanisms other than, or more likely in addition to, IL-1β synthesis prevention, and these are all questions that warrant future investigation.

Neonatal infection as a vulnerability factor

Collectively, we have demonstrated that neonatal infection in rats results in elevated glial cell markers and exaggerated IL-1β responses within the brain in adulthood, which appear to underlie memory impairment after an immune challenge. Neonatal infection appears to create a state of "vulnerability" for the animal that extends into adulthood, such that cognitive impairment occurs only after an immune challenge, the identical challenge having no impact in controls. There are interesting parallels between these data and observations within the aging and neurodegenerative disease literatures. For instance, chronic microglial activation is associated with many neuroinflammatory diseases involving cognitive deficits (e.g., Alzheimer's disease and AIDS-related dementia), and astrocyte reactivity in aged rats is positively correlated with cognitive impairment (Sugaya et al., 1996). Furthermore, cognitive impairment in aged or diseased populations during a subsequent infection or illness is generally more severe or prolonged compared with normal controls with the same infection/illness (Bodles and Barger, 2004; Perry, 2004). In future experiments, it will be important to examine the influence of early infection on different memory systems and cognitive abilities using a number of behavioral paradigms (e.g., spatial water maze and object recognition). In summary, an understanding of the mechanisms underlying neonatal infection-induced memory disruption may have implications for a variety of neuropsychiatric and neurodegenerative diseases.

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