



Environmental enrichment attenuates hippocampal neuroinflammation and improves cognitive function during influenza infection

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ARTICLE INFO

Article history:

Received 3 March 2012

Received in revised form 16 May 2012

Accepted 25 May 2012

Available online 9 June 2012

Keywords:

Influenza
Hippocampus
Cognition
Neuroinflammation
Cytokines
Microglia
Neurotrophins
Fractalkine
CD200

ABSTRACT

Recent findings from our lab have shown that peripheral infection of adult mice with influenza A/PR/8/34 (H1N1) virus induces a neuroinflammatory response that is paralleled by loss of neurotrophic and glial regulatory factors in the hippocampus, and deficits in cognitive function. Environmental enrichment has been shown to exert beneficial effects on the brain and behavior in many central nervous system (CNS) disorders, but its therapeutic potential during peripheral viral infection remains unknown. Therefore, the objective of the present study was to determine if long-term continuous exposure to environmental enrichment could prevent and/or attenuate the negative effects of influenza infection on the hippocampus and spatial cognition. Mice were housed in enriched or standard conditions for 4 months, and continued to live in their respective environments throughout influenza infection. Cognitive function was assessed in a reversal learning version of the Morris water maze, and changes in hippocampal expression of proinflammatory cytokines (IL-1 β , IL-6, TNF- α , IFN- α), neurotrophic (BDNF, NGF), and immunomodulatory (CD200, CX3CL1) factors were determined. We found that environmental enrichment reduced neuroinflammation and helped prevent the influenza-induced reduction in hippocampal CD200. These changes were paralleled by improved cognitive performance of enriched mice infected with influenza when compared to infected mice in standard housing conditions. Collectively, these data are the first to demonstrate the positive impact of environmental enrichment on the brain and cognition during peripheral viral infection, and suggest that enhanced modulation of the neuroimmune response may underlie these beneficial effects.

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1. Introduction

Influenza is a common and highly contagious viral pathogen that remains a significant health concern worldwide. Neurological symptoms have been reported following respiratory infection with pandemic strains of influenza A (CDC, 2009; Ravenholt and Foege, 1982; Studahl, 2003; Wang et al., 2010), suggesting that peripheral infection can impact central nervous system (CNS) function. While the mechanisms underlying the effects of non-neurotropic viral strains on the brain remain unclear (Schlesinger et al., 1998; Studahl, 2003), it has been hypothesized that glia-mediated neuroinflammation in association with common respiratory viruses may lead to cumulative neuronal damage across the lifespan (Majde, 2010).

Using a mouse model of influenza, we have recently shown that peripheral infection with influenza A/PR8/34 (H1N1) induces a

neuroimmune response, and impacts hippocampal structure and function (Jurgens et al., 2012). Infected mice demonstrated cognitive deficits in hippocampal-dependent tasks that were paralleled by significant alterations in hippocampal neuron morphology in the CA1 region and dentate gyrus. Concurrent with cognitive impairment, influenza-infected mice had increased hippocampal expression of inflammatory cytokines (interleukin (IL)-1 β , IL-6, tumor necrosis factor (TNF)- α and interferon (IFN)- α), and decreased expression of brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF). Previous work has shown that elevated levels of proinflammatory cytokines in the hippocampus can inhibit neurotrophic factors (Barrientos et al., 2004; Tong et al., 2008) and impair hippocampal synaptic plasticity (Lynch, 2002; Mendoza-Fernandez et al., 2000; Pickering and O'Connor, 2007), providing a potential mechanism by which hippocampal neuroinflammation can induce cognitive dysfunction. In addition, influenza significantly reduced the levels of immunomodulatory factors CD200 and CX3CL1 (fractalkine), which play an important role in the neuronal control of microglia activation (Cardona et al., 2006; Hoek et al., 2000), and have recently been found to impact synaptic development and integrity (Costello et al., 2011; Paolicelli et al., 2011; Ransohoff and Stevens, 2011). Overall, the loss of neurotrophic

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support along with dysregulated neuron-microglia crosstalk, could negatively impact cognitive function and leave the brain vulnerable to inflammatory damage during influenza infection.

A potential strategy for increasing brain plasticity and resiliency is environmental enrichment. Environmental enrichment (EE) is classically defined as a “complex combination of social and inanimate stimulation” (Rosenzweig and Bennett, 1996), in comparison to standard housing conditions. While there is variation in experimental protocols (Simpson and Kelly, 2011), in general EE refers to conditions that provide increased social, cognitive, and physical stimulation. An enriched environment for rodents typically includes group housing in a large cage with nesting materials, various toys, tunnels and ladders for exploration, and running wheels for voluntary exercise (Nithianantharajah and Hannan, 2006; van Praag et al., 2000). Items of enrichment are routinely changed during the experimental period to maintain novelty and provide continuous opportunity for exploration and learning.

Environmental enrichment has been shown to exert positive effects on cognitive and emotional behaviors including enhanced learning and memory (Bruehl-Jungerman et al., 2005; Leggio et al., 2005; Nilsson et al., 1999) and improved ability to cope with fear, anxiety and stress (Fox et al., 2006; Larsson et al., 2002; Schloesser et al., 2010). Early work demonstrated that exposure to a complex and stimulating environment could produce changes in neuron morphology, myelination and synaptogenesis during development, adulthood and aging (Bennett et al., 1969; Diamond et al., 1976; Green et al., 1983; Greenough et al., 1986; Juraska and Kopcik, 1988). This suggests that neural plasticity in response to environmental experience lasts throughout the lifespan. In addition to morphological changes, EE has also been shown to elevate levels of neurotrophic factors (Ickes et al., 2000; Pham et al., 1999) and synaptic proteins (Nithianantharajah et al., 2004; Rampon et al., 2000), as well as enhance hippocampal synaptic plasticity (Artola et al., 2006; Green and Greenough, 1986) and increase neurogenesis (Kempermann et al., 1997; Rossi et al., 2006).

While the beneficial effects of EE have been explored during aging, as well as in animal models of neurodegenerative disease, psychiatric disorders and brain injury (Laviola et al., 2008; Mora et al., 2007; Nithianantharajah and Hannan, 2006; Will et al., 2000), the impact of EE on the central immune response and cognitive behavior during peripheral viral infection remains unknown. Since influenza infection has been shown to negatively impact measures of hippocampal and cognitive function, which are influenced by EE, the present study sought to determine if the deleterious effects of influenza infection on the CNS could be prevented or attenuated by long-term continuous exposure to environmental enrichment. We examined the impact of environmental enrichment on influenza-induced sickness response and cognitive behavior, and assessed changes in inflammatory, neurotrophic, and immunomodulatory factors in the hippocampus. We focused our study on the hippocampus, as this is a brain region that demonstrates a high degree of plasticity, is vulnerable to inflammation, and plays an important role in cognitive function. We hypothesized that continuous exposure to an enriched environment throughout adulthood and during influenza infection would help modulate inflammation, preserve levels of neurotrophic and glial regulatory factors, and prevent or reduce cognitive deficits induced by peripheral infection with live influenza virus.

2. Materials and methods

2.1. Subjects

Young (6 weeks old) male BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). On arrival, mice were

group housed and allowed at least 7 days to acclimate before experimental housing treatments were initiated. Following acclimation, animals were randomly assigned to the enriched environment (EE, $n = 5\text{--}8/\text{cage}$) or standard environment (SE, $n = 1/\text{cage}$) conditions. Briefly, the enriched environment consisted of a large cage ($100 \times 51 \times 37$ cm) equipped with toys, tunnels, ladders, housing chambers, nesting material and two running wheels (Fig. 1). Mice were housed continuously in the enriched environment with rearrangement of toys, tunnels, and wheels 3–4 times per week to maintain novelty. The standard environment consisted of a laboratory polypropylene cage with bedding. All mice were maintained under a reverse 12:12 h light:dark cycle at 24 °C and given *ad libitum* access to food and water. All animal care and experimental procedures are in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and approved by the University of Illinois Institutional Animal Care and Use Committee.

2.2. Mouse viral infection

Mouse-adapted human influenza A/Puerto Rico/8/34 (H1N1) virus was a generous gift from Dr. John Sheridan and Dr. David Padgett (The Ohio State University). Mice were lightly anesthetized with isoflurane and intranasally (i.n.) inoculated with 1 HAU (hemagglutinating unit) of influenza A/PR/8/34 virus in 50 μ l sterile PBS. Control animals were inoculated (i.n.) with 50 μ l of sterile PBS. Use of isoflurane anesthesia allowed for quick recovery (<2 min) and accurate delivery of viral dose. Following viral infection, mice were monitored daily for signs of morbidity, including response to handling, food intake, and physical appearance (i.e. ruffled fur, huddled or hunched behavior). In mouse models of influenza, weight loss is considered a reliable indicator of disease progress (Matsuoka et al., 2009), thus body weight was measured at the same time daily (between 09:00 and 10:00) and percent change from initial body weight (day 0) was calculated. Previous studies (Lowder et al., 2005) have shown that during influenza infection, mice can lose a substantial amount of body weight (30–35%) and still recover, however as an established humane endpoint, mice that reached a 30% loss of their initial body weight were euthanized (day 7 post-inoculation).

2.3. Morris water maze

The effects of environmental enrichment and influenza infection on spatial learning and memory were assessed in a reversal learning version of the Morris water maze. In this hippocampal-dependent task, the animal must learn to use distinctive distal visuo-spatial cues surrounding the pool to navigate a direct path to the hidden platform (D'Hooge and De Deyn, 2001; Morris, 1984). The Morris water maze (MWM) consisted of a circular pool (130 cm diameter, 23–25 °C) with a transparent round platform (10 cm diameter) hidden 0.5 cm below the surface of the water. Mice were inoculated (day 0) and 2 days later began a 5-day acquisition phase (days 2–6 post-inoculation). The platform remained in the same location during the acquisition training. Animals were placed on the platform for 30 s preceding the start of each training session. The trials were conducted using a pseudorandom protocol in which mice were placed in the water in one of three preset entry locations. Mice were allowed to swim freely for 60 s or until the platform was reached. If the platform was not located during the 60 s, mice were guided to the platform and allowed to remain for 30 s. After completion of three consecutive trials, mice were placed in their home cage under a heat lamp to dry for 10 min. On day 7 post-inoculation (24 h after the last day of acquisition training), the platform was removed and mice received a 30 s probe trial to assess spatial memory for the platform location. Following the



Fig. 1. Enriched environment cage. Environmental enrichment consisted of social interaction ($n = 5\text{--}8$ mice in the cage), stimulation of exploratory behavior with objects such as toys and a set of tunnels, shelters, nesting materials, multiple locations of food and water *ad libitum*, and running wheels for voluntary exercise.

probe trial, mice were subjected to a reversal test in which the hidden platform was moved to the opposite quadrant of the pool, but all distal visual cues remained constant. Animals were placed on the platform for 30 s preceding the start of the reversal test, and given three trials to locate the platform in the new target quadrant. Reversal learning measures how quickly an animal is able to extinguish their initial learning of the platform's position and acquire a direct path to the new location (Vorhees and Williams, 2006). A video camera in conjunction with a computerized animal tracking system (Ethovision; Noldus Information Technologies) was used to record swim speed, latency to the platform, and distance swam (pathlength to the platform). For acquisition training and reversal testing, distance traveled was averaged across the three consecutive trials administered each day. Performance during the probe trial was measured as percent time in each quadrant location.

2.4. Quantitative real-time PCR

After completion of behavioral testing, mice were euthanized by CO_2 asphyxiation and blood and tissue samples were quickly collected and stored in RNAlater at -80°C . Total RNA was isolated from homogenized tissue using the Tri Reagent protocol (Sigma, St. Louis, MO). A QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA) was used for cDNA synthesis with integrated removal of genomic DNA contamination according to the manufacturer protocol. Quantitative real-time PCR was performed using the Applied Biosystems (Foster, CA) Assay-on-Demand Gene Expression protocol. In short, cDNA was amplified by PCR where a target cDNA (IL-1 β , IL-6, TNF- α , IFN- α , BDNF, NGF, CD200, CX3CL1 and influenza matrix M1) and a reference cDNA (glucose-3 phosphate dehydrogenase) were amplified simultaneously using an oligonucleotide probe with a 5' fluorescent reporter dye (6-FAM) and a 3' quencher dye (NFQ). PCR reactions were performed at the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. Fluorescence was determined on an ABI PRISM 7900HT-sequence detection system (Perkin Elmer, Forest City, CA). Data were analyzed using the comparative threshold cycle (Ct) method, and results are expressed as fold change (Schmittgen and Livak, 2008). For cytokines, neurotrophic

and immunomodulatory factors, results are expressed as fold change from standard-housed control mice. For M1 viral protein expression in the lung, results are expressed as fold change from lung tissue collected at day 1 post-inoculation as M1 was non-detectable in control (non-infected) tissue.

2.5. Experimental design

Mice remained in their respective housing (SE or EE) continuously for 4 months (7–8 weeks old to 6 months old). At 6 months old, mice were inoculated with influenza A/PR8/34 virus (1 HAU) or sterile PBS. Following inoculation, mice continued to live in their respective environments (SE or EE) throughout the entire post-inoculation period until they were euthanized for tissue collection at day 7 post-inoculation following behavioral testing. For each study, there were two enriched environment cages: one EE cage for control mice ($n = 5\text{--}7/\text{cage}$) and one EE cage for influenza-infected mice ($n = 5\text{--}7/\text{cage}$). For mice housed in the standard environment (SE), there were six cages of single-housed control mice, and six cages of single-housed influenza-infected mice. Therefore, a 2×2 factorial design was employed in this study: SE, standard environment; EE, enriched environment; CON, control (PBS inoculation); FLU, influenza (1 HAU viral inoculation), thus creating four treatment groups: SE CON, SE FLU, EE CON, and EE FLU. Using identical experimental parameters, two replications were performed, resulting in a final treatment number of $n = 10\text{--}12$ for all treatment groups.

2.6. Statistical analysis

Change in body weight, water maze acquisition, and probe trial data were subjected to repeated measures ANOVA as a two-way design in which housing (standard vs. enriched) and treatment (control vs. influenza) were between-subjects factors. The within-subjects factors were as follows: day post-inoculation (body weight), day of acquisition training (water maze acquisition), and water maze quadrant (probe trial). For expression of influenza M1 in the lungs, Student's *t*-test compared the means of SE FLU vs. EE FLU as M1 expression was non-detectable in the lungs of

control mice. All other data were analyzed using a 2-way ANOVA in which housing (standard vs. enriched) and treatment (control vs. influenza) were independent variables. When appropriate, differences between treatment means were evaluated with Fisher's protected least significant difference (PLSD) or Tukey–Kramer's multiple comparisons tests.

3. Results

3.1. Effects of environmental enrichment on influenza-induced sickness response

For body weight data (Fig. 2), repeated measures ANOVA revealed a day \times housing \times treatment ($F(6,39) = 3.5$, $p < 0.003$) interaction, indicating that influenza differentially affected body weight in SE and EE mice across days post-infection. While all influenza-infected mice lost weight over the course of infection (treatment \times day interaction $F(6,39) = 457.22$, $p < 0.0001$), post hoc analysis revealed that SE FLU mice lost more weight than EE FLU mice on days 5–7 ($p < 0.05$ for all), suggesting that environmental enrichment moderately attenuated the sickness response as the infection progressed.

3.2. Effects of environmental enrichment on influenza-induced cognitive impairment

In the Morris water maze (MWM), a decrease in time (latency) to find the hidden platform, or a decrease in pathlength (distance) to the platform, indicate an improvement in spatial learning. Since latency to reach the platform can be affected by motor function (swim speed) and motivation, distance swam is considered a more reliable measure of learning and memory performance (Contet et al., 2001; Cunningham and Sanderson, 2008), and therefore was used to determine the performance of mice during both acquisition training and reversal testing.

For the water maze acquisition data (Fig. 3A), repeated measures ANOVA revealed a significant effect of day ($F(4,36) = 15.04$, $p < 0.0001$) indicating that mice in all treatment groups showed improved performance (shorter pathlength) over days of acquisition training. The analysis also showed a main effect of housing ($F(1,36) = 14.02$, $p < 0.001$), indicating that mice in EE demonstrated superior performance to mice in SE and a main effect of

treatment ($F(1,36) = 4.26$, $p < 0.05$), with influenza infection impairing water maze performance to some extent in both SE and EE mice. The interaction of day \times housing \times treatment approached significance ($F(4,36) = 2.31$, $p = 0.06$), suggesting that influenza infection differentially altered the performance of SE vs. EE mice over acquisition training. On the last day of training (day 5), there was a significant housing \times treatment interaction ($F(1,36) = 4.8$, $p < 0.05$), with post hoc analysis showing that SE FLU mice were impaired compared to all other treatment groups ($p < 0.01$ for all comparisons). Overall the data suggested that enriched mice with influenza infection showed improved acquisition of the task when compared to influenza mice living in standard conditions.

For the probe trial (Fig. 3B), repeated measures ANOVA was employed to determine the effects of housing and influenza infection across time spent in each quadrant of the water maze. An interaction of housing \times quadrant ($F(3,32) = 3.38$, $p < 0.05$) indicated that quadrant preference during the probe trial differed for enriched and standard housed mice. Within each treatment group, differences in means were evaluated using Tukey–Kramer's procedure for multiple comparisons. For all treatment groups except SE FLU, there was a clear preference for the target quadrant ($p < 0.05$). Overall these data suggested that environmental enrichment helped prevent the influenza-induced impairment in spatial reference memory as assessed by probe trial performance.

For reversal testing data (Fig. 4), a 2-way ANOVA revealed a main effect of treatment ($F(1,30) = 6.03$, $p < 0.05$), but no effect of housing or housing \times treatment interaction, indicating that

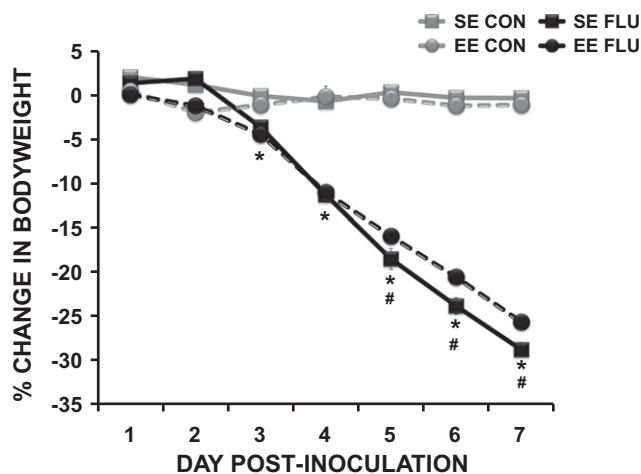


Fig. 2. Change in body weight following influenza infection. Influenza infection induced loss of body weight in both standard environment (SE) and enriched environment (EE) influenza mice. Influenza-infected mice in SE lost more body weight at day 5–7 post-inoculation than infected EE mice. Data are represented as means \pm SEM. (* $p < 0.05$ compared to control, # $p < 0.05$ SE FLU vs. EE FLU mice).

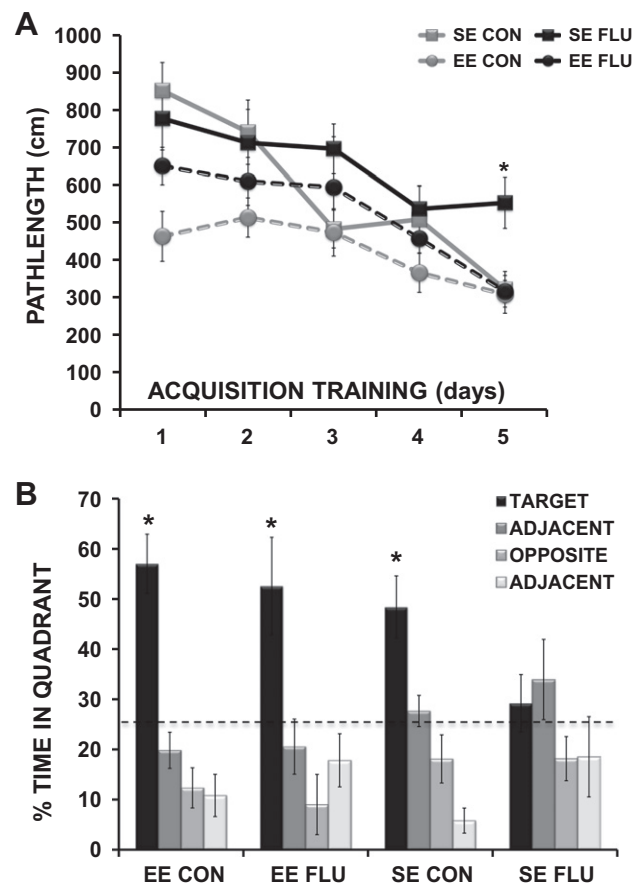


Fig. 3. Morris water maze acquisition and probe trial. (A) Distance to reach the hidden platform over 5 days of acquisition training (* $p < 0.01$ SE FLU vs. all other groups). (B) Quadrant occupancy during probe trial performed 24 h after the last day of acquisition training (* $p < 0.05$ target quadrant vs. non-target quadrants). Dashed line marks chance performance during testing.

influenza infection impaired reversal learning (as indicated by increased pathlength to the new platform location) in mice from both standard and enriched environments.

3.3. Effects of environmental enrichment and influenza on hippocampal cytokines

Overall, the analysis showed that there was no main effect of housing (standard vs. enriched) on the expression of IL-1 β , IL-6, TNF- α , IFN- α , or IFN- β ($p > 0.05$ for all), indicating that basal levels of hippocampal cytokines were not affected by living condition (Figs. 5 and 6). A housing \times treatment interaction for both IL-1 β ($F(1,35) = 6.88$, $p < 0.02$) and TNF- α ($F(1,34) = 5.89$, $p < 0.03$) indicated that expression of these proinflammatory cytokines following influenza infection was altered by environmental conditions (Fig. 5A,B). post hoc tests revealed that influenza-infected mice living in enrichment had significantly attenuated expression of both IL-1 β ($p < 0.05$) and TNF- α ($p < 0.05$) compared to standard housed infected mice. A significant main effect of treatment for IL-6 ($F(1,34) = 42.95$, $p < 0.0001$), IFN- α ($F(1,25) = 24.4$, $p < 0.0001$), and IFN- β ($F(1,36) = 25.35$, $p < 0.0001$) but no interaction between housing and treatment, indicated that influenza increased the expression of these cytokines to a similar extent in standard housed and enriched mice (Fig. 5C, Fig. 6A,B).

3.4. Effects of environmental enrichment and influenza on hippocampal neurotrophic and immunomodulatory factors

3.4.1. Neurotrophins

For BDNF expression (Fig. 7A), there was a main effect of housing ($F(1,35) = 22.16$, $p < 0.0001$) with enriched animals having increased hippocampal BDNF expression, and a main effect of treatment ($F(1,35) = 18.54$, $p < 0.0001$) indicating that influenza decreased BDNF in both enriched and standard housed mice. For NGF expression (Fig. 7B), there was a main effect of treatment ($F(1,35) = 38.04$, $p < 0.0001$), indicating that influenza decreased NGF in both standard housed and enriched mice. There was no main effect of housing on NGF expression ($p > 0.05$), indicating that unlike BDNF, environmental enrichment did not increase NGF expression in the hippocampus.

3.4.2. Immunomodulatory factors

For CX3CL1 (Fig. 7C), a main effect of treatment ($F(1,37) = 35.83$, $p < 0.0001$), indicated that influenza reduced CX3CL1 expression in

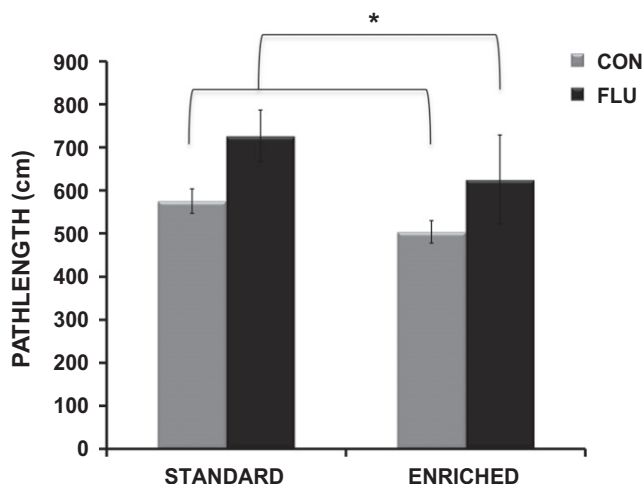


Fig. 4. Morris water maze reversal testing. Distance (pathlength) to reach the platform during reversal testing (average distance for 3 trials), $*p < 0.05$ influenza vs. control.

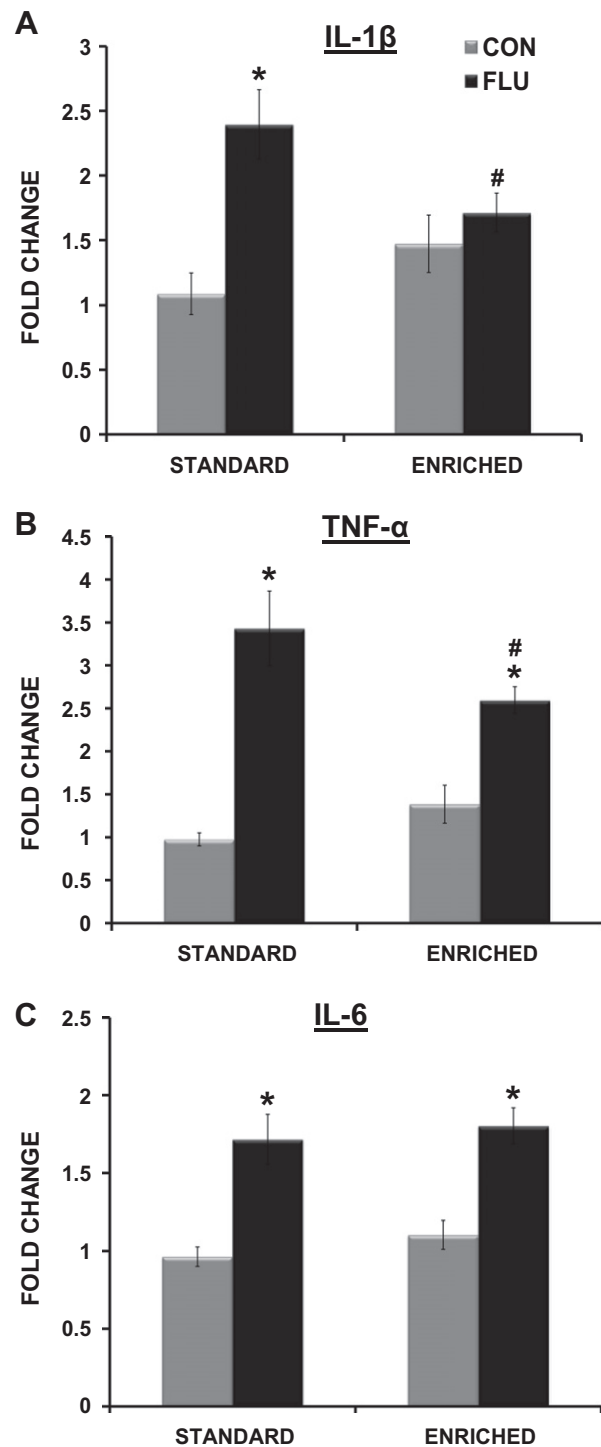


Fig. 5. Impact of environment and influenza infection on hippocampal proinflammatory cytokine expression at day 7 post-inoculation. Environmental enrichment attenuated the influenza-induced increased in IL-1 β (A) and TNF- α (B). Influenza infection induced IL-6 in both standard and enriched mice (C). $*p < 0.05$ compared to control, $#p < 0.05$ SE FLU vs. EE FLU.

mice from both environments. Interestingly, there was a main effect of housing ($F(1,37) = 8.19$, $p < 0.05$), in which environmental enrichment increased hippocampal CX3CL1 expression. For CD200 (Fig. 7D), the interaction of housing \times treatment approached significance ($F(1,37) = 3.65$, $p = 0.06$), with a main effect of treatment ($F(1,37) = 7.04$, $p < 0.05$) being driven by the decrease in CD200 expression in SE FLU mice. post hoc tests using Tukey–Kramer's procedure for multiple comparisons revealed that

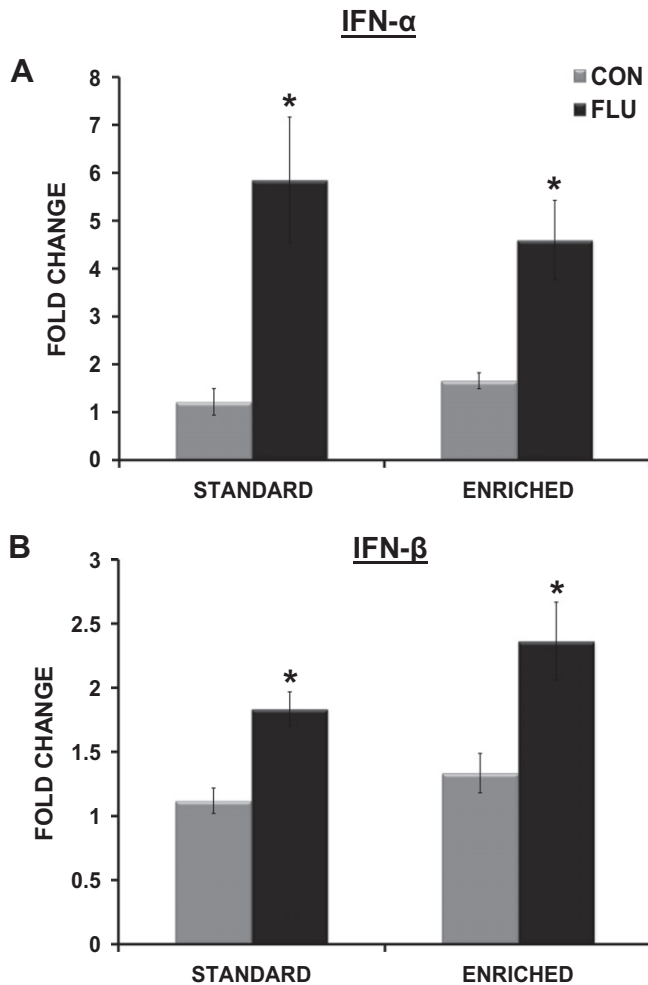


Fig. 6. Impact of environmental enrichment and influenza infection on hippocampal interferon cytokine expression at day 7 post-inoculation. Influenza infection induced IFN- α (A) and IFN- β (B) in both standard and enriched mice (* $p < 0.05$ compared to control).

while SE FLU mice had significantly decreased CD200 compared to controls ($p < 0.05$), expression of CD200 in the hippocampus of EE FLU mice was maintained at control levels ($p > 0.05$).

3.5. Effects of environmental enrichment on influenza viral infection and cytokine levels in the lungs

The data analysis indicated that on day 7 post-inoculation, influenza virus M1 expression was attenuated in the lungs of EE FLU mice when compared to SE FLU mice ($p < 0.01$) (Fig. 8A). Influenza M1 gene expression was non-detectable (ND) in the lungs of control mice. In addition, similar to our previous findings (Jurgens et al., 2012), there was no evidence of M1 gene expression in the hippocampus of control or infected mice (data not shown). A main effect of treatment for IL-1 β ($F(1,16) = 50.45$, $p < 0.0001$), IL-6 ($F(1,17) = 45.12$, $p < 0.0001$), TNF- α ($F(1,17) = 63.98$, $p < 0.0001$), IFN- α ($F(1,11) = 31.64$, $p < 0.0005$), and IFN- β ($F(1,16) = 62.59$, $p < 0.0001$) indicated that influenza infection increased the expression antiviral and inflammatory cytokines in the lungs of both EE FLU and SE FLU groups (Figs. 8 and 9).

4. Discussion

While the effects of environmental enrichment (EE) on the brain and behavior have been explored in healthy animals and in models

of neuropsychiatric disease and neurodegeneration (Laviola et al., 2008; van Praag et al., 2000), its potential therapeutic role during infectious disease remains less understood. In the present study, we determined if long-term exposure to EE would have beneficial effects on the brain and behavior in a mouse model of influenza infection. We found that environmental enrichment helped attenuate the sickness response and cognitive dysfunction associated with influenza infection. In addition, environmental enrichment buffered the influenza-induced alterations in hippocampal expression of inflammatory and immunomodulatory factors. These novel findings suggest that a non-invasive, non-pharmacological intervention such as environmental enrichment may help ameliorate the negative cognitive effects of peripheral infection. In regard to central function, this may be due to increased resiliency of the enriched brain to neuroinflammation, and/or the preservation of neuroimmune communication between neurons and microglia.

4.1. Environmental enrichment attenuates influenza-induced cognitive deficits

Overall, the data indicated the EE was moderately protective against influenza-induced deficits in hippocampal-dependent spatial learning and memory. Enriched influenza-infected mice showed improved performance during both acquisition training and the probe trial when compared to infected mice in standard housing conditions. Influenza-infected mice from both housing conditions were impaired during reversal testing, suggesting that when task difficulty is increased, or cognitive flexibility is needed, influenza-induced deficits are revealed. While hippocampal dysfunction can impede spatial reversal learning (Whishaw and Tomie, 1997), the frontal cortex plays an important role in the behavioral flexibility associated with reversal tasks (Kesner and Churchwell, 2011), and additional studies to determine the impact of influenza infection on this region are warranted.

In our previous study (Jurgens et al., 2012), we also found deficits during reversal learning in influenza infected mice, but did not find influenza-induced impairment during acquisition or the probe trial. This difference is likely attributed to the use of a larger maze in the present study. Since it has been shown that enriched mice demonstrate superior performance in the water maze (van Praag et al., 2000), a larger maze was utilized in the present study to prevent a “ceiling effect” from interfering with the detection of environmental or influenza-induced alterations in spatial learning and memory. Previous work has demonstrated that maze diameter significantly affects the acquisition and retention of spatial information in the water maze, and mice that perform well in smaller mazes, can demonstrate cognitive impairment when a larger maze diameter is employed (Van Dam et al., 2006; Vorhees and Williams, 2006). Overall, the results suggest that influenza-induced cognitive impairments are revealed when the difficulty of the task is increased, either by the use of larger maze or during reversal testing. Most importantly, influenza-infected EE mice demonstrated performance comparable to non-infected mice by the end of acquisition training and during the probe trial, suggesting that environmental enrichment may help to buffer the deleterious effects of influenza infection on hippocampal-dependent cognitive behavior.

4.2. Environmental enrichment attenuates influenza-induced hippocampal neuroinflammation

A potential contributing factor to cognitive detriment during influenza infection is hippocampal inflammation. While the central cytokine response to peripheral infection is essential for mounting an appropriate immune response and inducing adaptive sickness behaviors (Hart, 1988; Konsman et al., 2002), elevated or prolonged exposure to inflammatory mediators can have detrimental

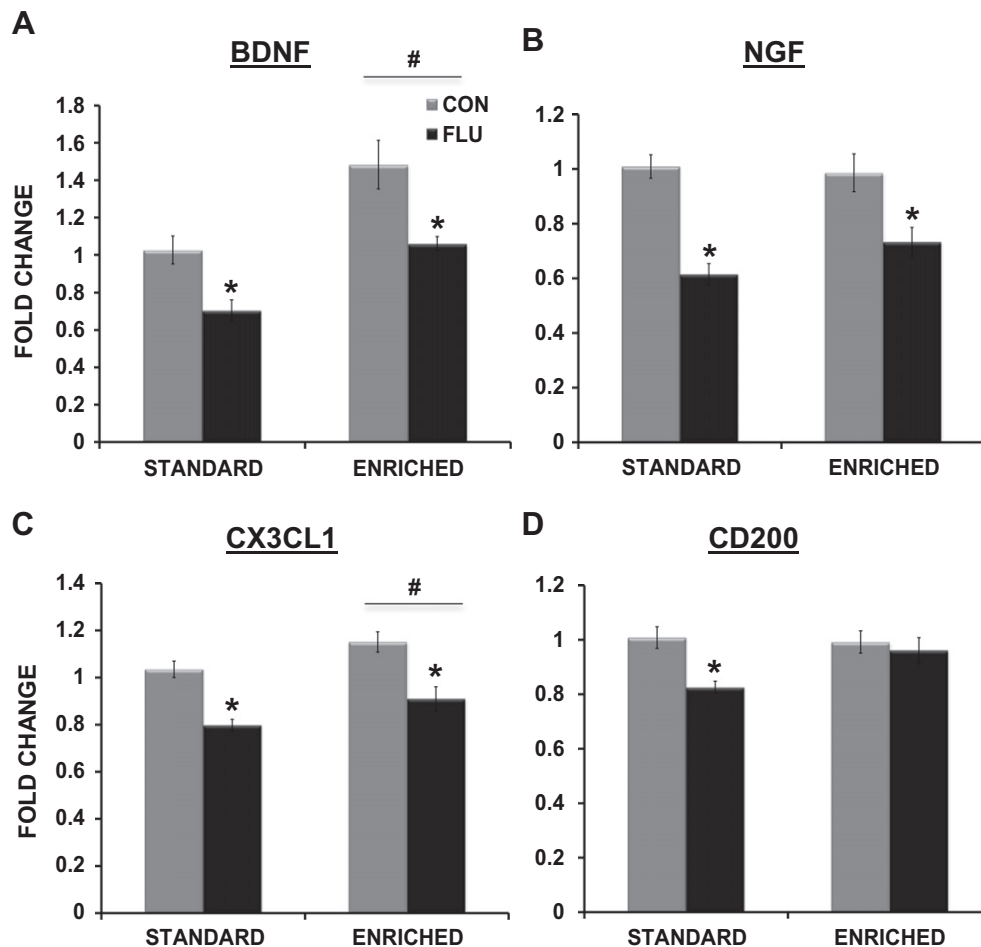


Fig. 7. Impact of environmental enrichment and influenza infection on hippocampal neurotrophic and immunomodulatory factor expression at day 7 post-inoculation. Environmental enrichment increased BDNF (A) and CX3CL1 expression (C). Influenza infection decreased the expression of BDNF, NGF and CX3CL1 in both enriched and standard housed mice (A,B,C). Environmental enrichment attenuated the influenza-induced decrease in CD200 (D). * $p < 0.05$ compared to control, # $p < 0.05$ standard vs. enriched housing.

effects on cognitive and emotional function (McAfoose and Baune, 2009; Yirmiya and Goshen, 2011). Similar to our previous findings, influenza infection increased expression of IL-1 β , IL-6, TNF- α and IFN- α in the hippocampus of standard housed mice. Most interestingly, environmental enrichment helped attenuate the influenza-induced increase in both IL-1 β and TNF- α expression. As IL-1 β in particular is known to specifically impact hippocampal-dependent tasks (Goshen et al., 2007; Hein et al., 2010), the EE-induced modulation IL-1 β expression could help preserve cognitive function during influenza infection. Additional neuroprotective effects of EE could be afforded by the attenuation of the influenza-induced increase in hippocampal TNF- α , which can impact synaptic plasticity, and at increased levels contribute to neurodegeneration (McCoy and Tansey, 2008; Pickering and O'Connor, 2007). As enrichment only partially attenuated IL-1 β and TNF- α , and did not prevent the increase in IL-6, IFN- α or IFN- β , which have also been shown to impact hippocampal function and cognition (Beyer et al., 2009; Sas et al., 2009; Sparkman et al., 2006), this may help explain why enriched mice only showed a partial rescue from cognitive deficits induced by influenza infection.

4.3. Effects of environmental enrichment on hippocampal expression of neurotrophic and immunoregulatory factors during influenza infection

As substantial evidence suggests that neurotrophic factors play an important role in learning and memory and hippocampal plasticity (Tyler et al., 2002; Waterhouse and Xu, 2009), the

influenza-induced reduction in hippocampal BDNF could have deleterious effects on cognitive function during influenza infection. While the enrichment paradigm increased basal levels of BDNF in the hippocampus, the expression of neurotrophic factors was blunted across housing conditions during influenza infection. This loss of neurotrophic support could help explain why even though enrichment improved cognitive function, deficits were still apparent during reversal testing in infected mice from both housing conditions. As recent work has shown that even low levels of neurotrophic overexpression in the hippocampus can attenuate epileptogenic neuroinflammation and prevent IL-1 β expression (Bovolenta et al., 2010), the higher “reserve” levels of BDNF in the hippocampus of enriched mice could provide neuroprotection and modulate inflammation upon immune challenge.

In addition to increasing BDNF, we also found that enrichment increased the expression of CX3CL1 (fractalkine), a chemokine constitutively expressed in the brain, that exerts immunomodulatory and anti-inflammatory effects during acute and chronic neuroinflammation (Cardona et al., 2006; Harrison et al., 1998; Lyons et al., 2009; Zujovic et al., 2001). While well-known for its role in immune function, recent evidence suggests that disruption of CX3CL1 signaling can inhibit neurogenesis (Bachstetter et al., 2011), impair microglia-mediated synaptic pruning in the hippocampus (Paolicelli et al., 2011), and prevent the enhancement of neuronal plasticity and spatial cognition induced by environmental enrichment (Maggi et al., 2011), suggesting it may play an important role in hippocampal function. The loss of CX3CL1 during

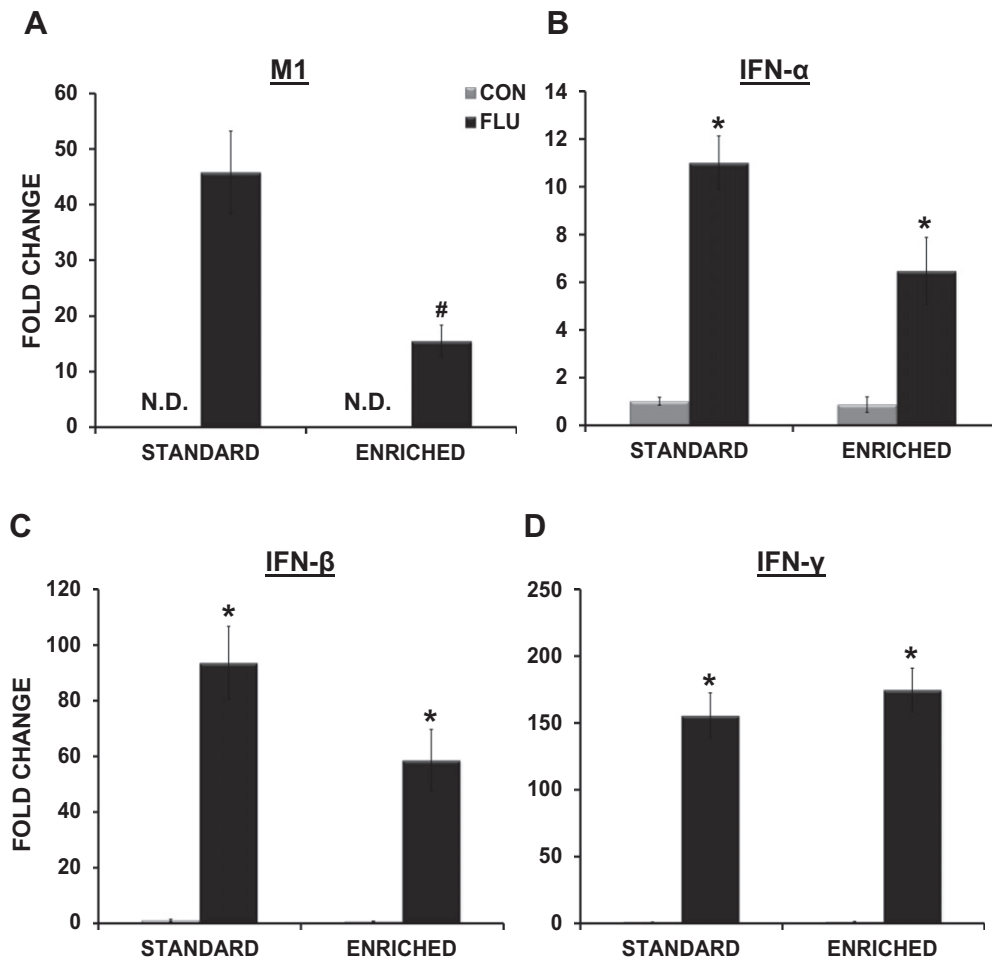


Fig. 8. Impact of environmental enrichment and influenza infection on expression of influenza virus and cytokines in the lung at day 7 post-inoculation. (A) Environmental enrichment attenuated the expression of influenza matrix (M1) protein. M1 expression was non-detectable (N.D.) in control samples. Influenza infection induced IFN- α (B) and IFN- β (C) and IFN- γ (D) in both standard and enriched mice. * $p < 0.05$ compared to control, # $p < 0.05$ SE FLU vs. EE FLU.

influenza infection could contribute to changes in both glial regulation and cognitive function. While enrichment was not effective in preventing the influenza-induced decrease in CX3CL1, it is possible that the prior EE-induced increase CX3CL1 could set the stage for constrained induction of neuroinflammation and better maintenance of neuronal structural and synaptic plasticity during viral infection.

The ability of EE to attenuate the influenza-induced decrease in CD200 could be an important factor in maintaining essential neuron-microglia interactions during influenza infection. While it is known that CD200 is important for keeping microglia in a resting state in both the healthy and diseased brain (Chitnis et al., 2007; Hoek et al., 2000), recent evidence suggests that constitutive expression of CD200 is essential for normal hippocampal plasticity and resiliency from inflammatory stimuli (Costello et al., 2011). In addition, it was recently shown that hippocampal dendrites express high levels of CD200, suggesting a role for this immune mediator in glial-synaptic interactions (Ojo et al., 2011). As we have previously shown that influenza infection induces hippocampal dendritic atrophy (Jurgens et al., 2012), this could contribute to decreased expression of CD200 in the hippocampus of standard housed infected mice. While future work is needed to determine the influence of EE on neuronal architecture during influenza infection, it is possible that enrichment could reduce neuronal atrophy and help preserve CD200 levels in the hippocampus of influenza-infected mice. Overall, the maintenance of physiological levels of

CD200 in the hippocampus of enriched mice during influenza infection may reduce neuronal vulnerability to inflammation and help maintain neuron-glia interactions important in synaptic function.

4.4. Effect of environmental enrichment on viral infection and immune response in the lungs

While environmental enrichment reduced proinflammatory cytokine production in the brain during influenza infection, there was no effect of EE on the expression of inflammatory or anti-viral cytokine production in the lungs of infected mice. However, enrichment did reduce influenza viral load (as measured by influenza M1 expression in the lungs), and attenuate the loss of body weight over the course of infection, suggesting that enriched mice were better able to cope with the viral infection. While not addressed in the current study, it has been shown that EE can alter NK and T cell activity (Benaroya-Milshtein et al., 2004; Marashi et al., 2004) which could influence the immune response during infection, as these cell populations play a vital role in the control of influenza viral infection (Culley, 2009; Kim et al., 2011).

In addition, previous work has demonstrated that moderate exercise following infection with influenza virus reduced lung cellular infiltrate and pathology, and increased survival in adult mice (Lowder et al., 2006). Similar to our findings, these studies found there to be no reduction in lung IL1- β or TNF- α expression, but

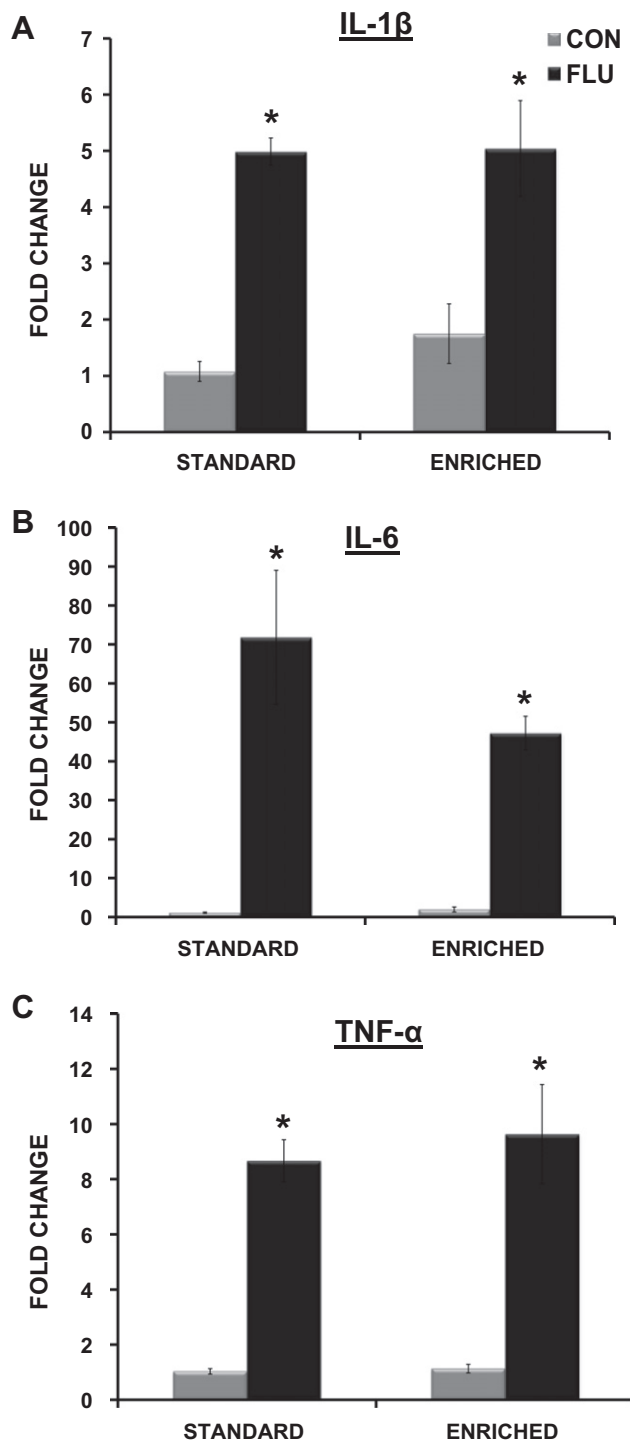


Fig. 9. Impact of environmental enrichment and influenza on expression of inflammatory cytokines in the lung at day 7 post-inoculation. Influenza infection induced IL-1 β (A), IL-6 (B) and TNF- α (C) in both standard and enriched mice. * $p < 0.05$ compared to control.

interferon levels were reduced, and there was tendency toward reduction of influenza M1 expression at day 3 and 5 post-infection. Taken together, these findings suggest it is likely that the effects of increased physical activity afford some portion of the beneficial impact of EE during influenza infection. Indeed, exercise has been shown to modulate both the peripheral and central immune response (Packer et al., 2010; Walsh et al., 2011), as well as exert positive effects on brain morphology, plasticity, and cognition (Cotman et al., 2007; van Praag, 2009). Furthermore, a recent study

found exercise to be the main factor mediating increased BDNF levels and hippocampal neurogenesis in the hippocampus of environmentally enriched mice (Kobilo et al., 2011). While additional studies could determine the extent to which individual components of enrichment (i.e. physical activity, social or cognitive stimulation) effect the peripheral vs. central immune response to influenza infection, evidence suggests that at least in terms of the brain, the greatest benefits are gained from additive or synergistic effects of the full repertoire of environmental enrichment (Faherty et al., 2003; Leggio et al., 2005; Sozda et al., 2010). It is also important to point out that while the majority of studies investigating the neural effects of EE use single-housed animals as the control group (Simpson and Kelly, 2011), there is evidence that paradigms of “social isolation” can induce adverse immune and behavioral effects (Lu et al., 1999; Voikar et al., 2005). Further studies are warranted to investigate the effects of individual vs. social housing on neuroimmune and cognitive parameters during influenza infection. While EE may provide resiliency toward influenza infection, the possibility that isolation could potentiate the effects of peripheral viral infection is an important and interesting question that requires further exploration.

4.5. The impact of environmental enrichment on the neuroimmune response

While the beneficial effects of environmental enrichment have primarily focused on changes in neuronal morphology, synaptic plasticity, and neurogenesis, EE also induces changes in non-neuronal components including cerebrovasculature, oligodendrocytes, and astrocytes (Markham and Greenough, 2004; Soffie et al., 1999; Viola et al., 2009). While less is known regarding the effects of EE on microglia, evidence suggests that they are also responsive to physical and cognitive stimulation. Previous work has found that enriched adult rats had increased numbers of microglia in the dentate gyrus that displayed a phenotype associated with neuroprotection and enhanced neurogenesis (Ziv et al., 2006). In a model of neurodegeneration, EE was shown to shift microglia from a neurotoxic to phagocytic phenotype that was associated with reduced amyloid burden, upregulation of anti-inflammatory factors, and improvements in learning and memory (Dong et al., 2007). Overall these data suggest that environmental enrichment can promote a microglia phenotype associated with reduced inflammation and increased neuroprotection in both health and disease.

While these exciting findings suggest that microglia are sensitive to enriched conditions, there is very little data regarding the impact of EE on the central immune response to infectious disease. Previous work has investigated the effects of EE in models of direct CNS infection, and while one study found no benefit on neurogenesis or spatial learning when implemented after infection with bacterial meningitis (Tauber et al., 2009), another study found mice reared in EE to have less viral neuroinvasion, reduced microglial activation, and improved behavioral outcome in a model of neurotropic viral encephalitis (de Sousa et al., 2011). In addition, it was recently shown that exposure to EE altered glial phenotype and attenuated the proinflammatory response in the hippocampus of rats injected peripherally LPS (Williamson et al., 2012). Overall these data provide evidence that the effects of immune activation on the brain and behavior are shaped by environmental conditions.

To our knowledge the present study is the first to implement EE during peripheral viral infection thus extending the beneficial effects of environmental enrichment on CNS function to a highly pathogenic and clinically relevant respiratory disease. The ability of enriched mice to better modulate the brain's response to peripheral infection could contribute to more efficient maintenance of CNS homeostasis during influenza infection, thus allowing for improved preservation of cognitive function. The protective effects of

EE during influenza infection may be related to the development of brain and cognitive reserve, in which a physically and mentally active lifestyle promotes brain resiliency through more efficient and flexible networks that are less susceptible to disruption induced by injury or neurodegeneration (Petrosini et al., 2009). While environmental enrichment itself is a simple, non-invasive therapeutic that can provide resiliency and reserve during brain insult, injury or inflammation, the elucidation of the mechanisms underlying the positive effects of environmental enrichment could help in the implementation of more specific interventions aimed at protecting neural function during peripheral infection.

Acknowledgement

This research was supported by National Institutes of Health Grant AG016710 (R.W.J.).

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