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Sleep fragmentation and sepsis differentially impact blood–brain barrier integrity and transport of tumor necrosis factor- α in aging

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Abstract

The factors by which aging predisposes to critical illness are varied, complex, and not well understood. Sepsis is considered a quintessential disease of old age because the incidence and mortality of severe sepsis increases in old and the oldest old individuals. Aging is associated with dramatic changes in sleep quality and quantity and sleep increasingly becomes fragmented with age. In healthy adults, sleep disruption induces inflammation. Multiple aspects of aging and of sleep dysregulation interact *via* neuroimmune mechanisms. Tumor necrosis factor- α (TNF), a cytokine involved in sleep regulation and neuroimmune processes, exerts some of its effects on the CNS by crossing the blood–brain barrier (BBB). In this study we examined the impact of sepsis, sleep fragmentation, and aging on BBB disruption and TNF transport into brain. We used the cecal ligation and puncture (CLP) model of sepsis in young and aged mice that were either undisturbed or had their sleep disrupted. There was a dichotomous effect of sepsis and sleep disruption with age: sepsis disrupted the BBB and increased TNF transport in young mice but not in aged mice, whereas sleep fragmentation disrupted the BBB and increased TNF transport in aged mice, but not in young mice. Combining sleep fragmentation and CLP did not produce a greater effect on either of these BBB parameters than did either of these manipulations alone. These results suggest that the mechanisms by which sleep fragmentation and sepsis alter BBB functions are fundamentally different from one another and that a major change in the organism's responses to those insults occurs with aging.

Keywords

Sleep; Aging; Sepsis; Tumor necrosis factor- α ; Blood–brain barrier

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1. Introduction

The neuroimmunology of aging is an understudied area that is important to many aspects of health in individuals over age 65. Aging is associated with increased susceptibility to disease, and increased prevalence of morbidity and mortality. The most recent census data indicate that the proportion of individuals older than age 65 is increasing at an unprecedented rate. This shifting demographic is reflected in the hospital setting, where half the ICU admissions and more than half the total number of ICU days are accounted for by older adults (Milbrandt et al., 2010; Pisani, 2009). For most traumas, including sepsis, morbidity and mortality are much greater in the old and oldest-old populations. Patients over the age of 65 constitute 65% of the sepsis patients (Martin et al., 2006), and a history of a septic event greatly increases the rate of all-cause mortality in the elderly (Angus et al., 2001; Weycker et al., 2003). Recent studies suggest that increased morbidity and mortality in older people may be due to a chronic inflammatory state, so called “inflammaging” (Giunta, 2008; Hunt et al., 2010a,b).

Aging is characterized by dramatic changes in sleep (Ancoli-Israel, 2009; Roffwarg et al., 1966). Total daily sleep time is reduced and sleep becomes increasingly disrupted with aging (Ancoli-Israel, 2009; Bliwise, 1993; Edwards et al., 2010). Sleep disruption of healthy young and older adults is associated with increased production of inflammatory mediators (Frey et al., 2007; Haack et al., 2007; Irwin et al., 2006; Mullington et al., 2009; Simpson and Dinges, 2007; Vgontzas et al., 2003), including the cytokine tumor necrosis factor- α (TNF). As such, sleep disruption in the elderly may contribute to, or exacerbate sepsis outcomes because of the inflammatory state it induces.

Sepsis outcomes and sleep fragmentation are linked by neuroimmune components. For example, TNF promotes non-rapid eye movement sleep (NREMS) after central or peripheral administration (Imeri and Opp, 2009; Kapas et al., 1992; Krueger et al., 2011; Zielinski et al., 2013), TNF mRNA and protein are highest during sleep (Bredow et al., 1997; Floyd and Krueger, 1997), and mice lacking the TNF p55 receptor spend less time NREMS (Feng et al., 1997). To reach its CNS receptors, blood-borne TNF must cross the blood–brain barrier (BBB) and it does so by a saturable transport system (Gutierrez et al., 1993). This transporter, in turn, is altered by some neuroimmune events, but not by others. For example, the TNF transport rate across the BBB is greatly enhanced in experimental autoimmune encephalomyelitis (Pan et al., 1996), but not with administration of lipopolysaccharide (Osburg et al., 2002). However, lipopolysaccharide has other effects on the BBB, including disrupting its integrity (Allen, 1965; Eckman et al., 1958).

Because aging is characterized by changes in many processes and systems that may contribute to increased morbidity and mortality, in this study we examine the interactive effects of aging, sepsis, and sleep fragmentation on BBB integrity and transport of TNF across the BBB. We use a clinically-relevant model of sepsis, cecal ligation and puncture (CLP), because it reproduces the hemodynamic changes of sepsis, recreates the progressive release of proinflammatory mediators, and is responsive to antibiotics and fluid resuscitation (Nemzek et al., 2008; Newcomb et al., 1998). We now report that sleep fragmentation and sepsis differentially impact BBB integrity and transport of TNF in an age-associated manner.

2. Methods

2.1. Mice

All studies were approved by the University of Washington Institutional Animal Care and Use Committee (IACUC), in accordance with the US Department of Agriculture Animal Welfare Act and the National Institutes of Health policy on Humane Care and the Use of Laboratory Animals. Male C57BL/6 mice aged 20 month were obtained from the National Institute of Aging aged rodent colony program. Aged mice in this study were provided from the colony at Charles River Laboratories International, Inc., (Wilmington, MA). Young (3 month) C57BL/6 mice were purchased from Charles River Laboratories International, Inc., to assure the same genetic background for both age groups. Mice were group housed until surgery (see later), and maintained on a 12:12 h light:dark cycle at 27 ° C. All mice had *ad libitum* access to standard laboratory chow and water.

2.2. Iodination of TNF and albumin

Murine TNF purchased from R&D Systems, Inc., (Minneapolis, MN) was radioactively labeled with ^{125}I by the lactoperoxidase method and the resulting ^{125}I -TNF (I-TNF) was purified on a column of G-10 Sephadex. In brief, 5 microg of TNF was reacted with 1 mCi of radioactive iodine and eluted with chloride free phosphate buffer. Specific activity of the I-TNF was approximately 700 Ci/mmol. Bovine albumin purchased from Sigma-Aldrich (St. Louis, MO) was labeled with ^{131}I by the chloramine-T method and the resulting ^{131}I -albumin (I-Alb) purified on a column of G-10 Sephadex as above. Both the I-TNF and I-Alb appeared in the void volume fractions.

2.3. Cecal ligation and puncture

Polymicrobial intraabdominal infection was induced by CLP as previously described (Remick et al., 2000). Briefly, mice were anesthetized with isoflurane and the cecum exposed and ligated with 4.0 prolene below the ileocecal junction, approximately 1.5 cm from the distal end. The cecum was then twice punctured through-and-through with a sterile 21-gauge needle and compressed to extrude fecal contents. For those mice that served as controls, a SHAM surgery was performed. During SHAM (control) surgeries, the cecum was exposed in the same manner as during CLP surgeries, but was not ligated or punctured. All mice received a broad spectrum antibiotic (Imipenem) in 1 ml lactated Ringers/D5W subcutaneously 2 h post-surgery and twice daily. Antibiotics are generally administered to rodents used in CLP models of sepsis because antibiotic treatment increases the number of animals that survive (Newcomb et al., 1998). Furthermore, human patients who become septic are treated with antibiotics, and CLP has been used as a model to determine the effectiveness of antibiotics as a therapeutic intervention. In this study, we administered antibiotics to reduce mortality so that sufficient numbers of mice would survive for study. To account for the impact of antibiotics per se, control (SHAM) mice also received the same antibiotic treatment.

2.4. Sleep fragmentation

The sleep fragmentation device used in this study has been validated and known to effectively disrupt sleep of mice for prolonged periods (Ringgold et al., 2013). The device consists of a cylindrical Plexiglass® chamber divided into two separate compartments. The floor of the chamber is a disc that is programmed to rotate at specific intervals. In this study, disc rotations were confined to the 12 h light period, and consisted of an 8 s rotation once every 30 s, on average. The direction of the disc rotation was randomized, and the precise placement of the 8 s rotation within the 30 s period was varied to prevent behavioral adaptation by a mouse to disc movements. Sleeping mice wake up when the disc rotates, whereas awake mice continue their normal behavior. During the 12 h dark period there was no disc rotation, and mice were allowed spontaneous behavior. Mice were housed in the sleep disruption devices, one mouse in each compartment, for 3 days of habituation prior to beginning the sleep fragmentation protocol.

2.5. TNF transport into brain

At specific time points (see later), mice were anesthetized with intraperitoneal urethane (4.0 g/kg) and the left jugular vein and right carotid artery exposed. Ringer's lactated solution (LR; 0.2 ml) with 1% bovine serum albumin (BSA) containing 10^6 cpm of I-TNF and 10^6 cpm or I-Alb was injected into the jugular vein. Blood was collected from the carotid artery and these samples were centrifuged at 5000g for 10 min at 4 °C and the serum collected. Whole brains were cleaned of large vessels and weighed after discarding the pituitary and pineal gland. Radioactivity in brain and arterial serum was measured in a gamma counter and brain/serum ratios (μ l/g) calculated. Values for I-TNF were corrected for vascular space (delta I-TNF) by subtracting the brain/serum ratio for I-Alb.

Multiple-time regression analysis (Blasberg et al., 1983a,b) was used to calculate the blood to brain unidirectional influx rate (K_i). The brain/serum ratios were plotted against their respective exposure times. Exposure time (Expt) was calculated from the formula:

$$\text{Expt} = \left[\int_0^t C_p(\tau) d\tau \right] / C_{pt}$$

where C_p is radioactive counts in serum and C_{pt} is radioactive counts in serum at time t . Expt corrects for the clearance of peptide from the blood. K_i with its error term is measured as the slope for the linear portion of the relation between the brain/serum ratios and Expt, and the Y intercept of the linearity measures V_i , the distribution volume in brain at $t = 0$, so that the equation describing the linear portion of the relation between brain/serum ratios and Expt is:

$$\text{brain/serum ratio} = K_i(\text{Expt}) + V_i.$$

2.6. Statistics

Linear regression was performed by the least squares method and the slopes of the lines (the K_i 's) compared using the Prism 6.0 statistical package (GraphPad, Inc., San Diego, CA).

Two means were compared by t-test. More than two means in one category were compared by one-way analysis of variance (ANOVA) followed by Newman–Keuls post-test. More than two means in two categories were compared by two-way ANOVA followed by the Bonferroni post-test (Prism 6.0). Statistical significance was taken as $p < 0.05$.

2.7. Experiment 1. Time course for sepsis-induced alteration of TNF transport across the BBB in young mice

Acute responses to sepsis induction in this model manifest during the first 1–3 days after CLP surgery, with 30–40% mortality occurring 3–4 days post CLP (Granger et al., 2013). To determine an appropriate time point at which to obtain measures of TNF transport across the BBB, *young* mice were subjected to SHAM or CLP surgeries and single time point transport of TNF across the BBB determined 24 h or 48 h later ($n = 5$ for each group for a total of 20 mice in this study). These two times were selected because most mortality occurs after this time. These mice were not subjected to sleep disruption, and served to inform as to sampling times to use in the full protocol that includes both age groups and manipulations of sleep. For this experiment, I-Alb and I-TNF were injected into the jugular vein and brain and blood samples collected 5 min later.

2.8. Experiment 2. Effects of aging on sepsis-induced alterations in BBB integrity and TNF transport

To characterize the nature of the changes in the BBB, measures of steady state and influx rate of I-TNF and I-Alb transport into brain were determined. A total of 36 mice were used in this study. Based on the results of experiment 1, these studies were conducted 24 h after CLP. Influx rate (K_i , multiple-time regression analysis) was determined from serial blood samples taken from each animal at multiple time points between 1 and 10 min after the intravenous injection of I-Alb and I-TNF.

2.9. Experiment 3. Effects of aging and sleep fragmentation on sepsis-induced alterations in BBB integrity and TNF transport

The effects of sleep disruption and aging on BBB disruption and TNF transport and across the BBB were determined in a separate group of mice. A total of 75 mice were used in this experiment. After 3 days habituation to the sleep disruption devices, aged and young mice were randomized into undisturbed or sleep fragmented (SF) groups. Undisturbed animals were housed in the sleep disruption devices, but no disc rotations occurred, whereas daytime sleep of mice in the SF groups was disrupted as detailed in Section 2.4. During the next light period, all mice were then further randomized into groups that received either SHAM or CLP surgeries. As such, mice in the SF groups were allowed undisturbed, *ad lib* behavior for 12-h prior to SHAM or SLP surgeries. After surgeries, mice were observed until ambulatory and then were placed back into the sleep disruption devices, but no further sleep disruption occurred. At time of study, the 8 groups of mice were distributed as follows:

Young, sham ($n = 10$)

Young, CLP ($n = 9$)

Young, SF ($n = 10$)

Young, CLP + SF ($n = 10$)

Aged, sham ($n = 10$)

Aged, CLP ($n = 10$)

Aged, SF ($n = 8$)

Aged CLP + SF ($n = 6$)

Based on results of Experiment 1, which revealed that TNF across the BBB was the same 24 h or 48 h after CLP, all mice in this experiment were sacrificed 24 h after CLP or SHAM surgeries.

3. Results

3.1. Experiment 1. Time course for sepsis-induced alteration of TNF transport across the BBB in young mice

An abbreviated time course of I-TNF transport across the BBB was determined from samples obtained 24 h and 48 h after CLP or SHAM surgeries in 2 month old mice. CLP significantly increased TNF transport across the BBB at 24 h and 48 h post-surgery (Fig. 1). There was no difference in the amount of I-TNF detected in brain between these two time points. Based upon these results, other measures of BBB transport and integrity were obtained 24 h after CLP or SHAM surgeries.

3.2. Experiment 2. Effects of aging on sepsis-induced alterations in BBB integrity and TNF transport

The brain/serum ratios did not vary with time for I-Alb in aged, young, sham, or CLP groups. This allowed combining the results without respect to time to obtain a single measure of the albumin space in brain. A t-test showed a significant increase in the brain/serum ratios for young mice (Fig. 2, upper left panel, $t = 3.87$, $df = 17$, $p < 0.01$), but not in aged (Fig. 2, upper right panel) mice. Brain/serum ratios for TNF correlated with time, indicating that TNF was crossing the BBB. In young mice (Fig. 3 lower left panel), CLP significantly increased the rate at which TNF crossed the BBB from a rate of 0.499 microl/g-min to 3.37 microl/g-min ($p < 0.05$). In aged mice (Fig. 3, lower right panel), the rate of TNF transport across the BBB was not significantly affected by CLP, although the intercept of the line was significantly decreased ($p < 0.05$).

3.3. Experiment 3. Effects of aging and sleep fragmentation on sepsis-induced alterations in BBB integrity and TNF transport

The complex interactions and effects of age, sleep fragmentation, and CLP on BBB integrity and TNF transport are depicted in Fig. 3. In *young mice*, CLP but not sleep fragmentation altered BBB integrity, as evidenced by increased the I-Alb space (Fig. 3, upper left panel). In addition, CLP in combination with sleep fragmentation in young mice did not alter I-Alb beyond values obtained from mice that had only CLP. In contrast, I-Alb increased in *aged mice* subjected to sleep fragmentation, but not CLP (Fig. 3, upper right panel), and there was no further increase in I-Alb when CLP was combined with sleep fragmentation. Aged mice had higher values for I-Alb brain/serum ratios than young animals for the sham and sleep

fragmentation groups, but values did not differ between young and aged for the CLP or CLP plus sleep fragmentation groups.

There were differential effects of CLP and sleep fragmentation on BBB transport of TNF. CLP, but not sleep fragmentation, increased TNF transport in young mice (Fig. 3, lower left panel), and CLP plus sleep fragmentation did not increase TNF transport beyond that of CLP alone. In *aged animals*, sleep fragmentation, but not CLP, increased TNF transport (Fig. 3, lower right panel), and CLP plus sleep fragmentation did not increase transport any more than sleep fragmentation alone.

4. Discussion

These studies examined the effects and interactions of aging, sepsis as induced by CLP, and sleep fragmentation on the integrity of the BBB and on its ability to transport TNF. BBB integrity is essential to the normal functioning of the CNS, and TNF transport across the BBB correlates with CNS effects (Joshi et al., 2010; Qin et al., 2007). Transport of TNF across the BBB is modulated in states that are associated with inflammation and neuroinflammation such as spinal cord injury (Pan et al., 1997; Pan and Kastin, 2001), experimental autoimmune encephalomyelitis (Pan et al., 1996), traumatic brain injury (Pan et al., 2003), and stroke (Pan et al., 2006), but transport is not increased by treatment with lipopolysaccharide (Osburg et al., 2002).

Here, we found that in young mice CLP disrupts the BBB to albumin and increases the transport rate of TNF. TNF circulates as a trimer that is nearly the same size as albumin. Despite this, both the baseline and CLP-stimulated rate of passage across the BBB for TNF is approximately 40 times faster than that previously reported for albumin (Gutierrez et al., 1993). In this study, the albumin space is increased by 21% in young mice subjected to CLP. This increase in albumin space is likely due mainly if not entirely to disruption of the BBB, although a contribution of vasodilation was not ruled out. In contrast, the rate of TNF transport across the BBB increased nearly 7-fold. This effect of CLP was sustained for at least 48 h, as shown in Fig. 1.

The degree of disruption found here was small, especially in comparison to the large increase in TNF transport. Smaller agents such as sucrose are often used to measure BBB disruption and smaller agents are usually more sensitive in detecting BBB disruptions as illustrated under many conditions (Huber et al., 2006; Ziylan et al., 1984). However, albumin was chosen as it is nearly the same size as trimeric TNF and so served not only to measure BBB disruption, but also as a kind of comparative control to TNF.

Although disruption of the BBB and increased transport of TNF across the BBB are both associated with neurotoxic events, they likely have very different implications for brain function. Disruption of the BBB occurs in a variety of diseases associated with inflammation, which is thought to be the major driver of that disruption. Besides sepsis, these conditions include multiple sclerosis (Pozzilli et al., 1988), neuroAIDS (Avison et al., 2004), diabetes mellitus (Huber et al., 2006), traumatic brain injury (Baldwin et al., 1996; Higashida et al., 2011), stroke (Abraham et al., 2003), dementias associated with

Alzheimer's disease and vascular disease (Erickson and Banks, 2013), and seizures (Oztas et al., 1990). BBB disruption, even when modest, results in neuronal degeneration secondary to the influx of blood-borne substances that are neurotoxic (Bowyer and Ali, 2006).

Transport of TNF across the BBB is much more nuanced. TNF has several actions directly on the BBB, including disruption (Deli et al., 1995; Fiala et al., 1997). Other actions, however, are consistent with physiological regulation of BBB function by TNF, including regulation of the efflux transporter p-glycoprotein (Yu et al., 2008, 2007) and immune cell trafficking (Mason and Van Epps, 1989). TNF transport across the BBB is dependent on both the p55 and p75 TNF receptors (Pan and Kastin, 2002) and is altered in various models of CNS injury (Barten and Ruddle, 1994; Pan et al., 1997, 1996) or disease (Banks et al., 2001). TNF transport has been specifically implicated in mediating neurotoxicity in models of Parkinson's disease and "chemobrain" (Kenney et al., 2013; Qin et al., 2007).

In aged mice, however, neither BBB disruption nor TNF transport increased after CLP. This resistance of the aged BBB to change is surprising as the aged BBB is usually thought to be more vulnerable to insults than that of younger animals. The explanation for this lack of response of the BBB is not clear, but could be from an age-related decrease in immune response, due to an age-related leaky gut inducing LPS tolerance, or other phenomena. In contrast, the aged BBB is susceptible to sleep fragmentation as BBB disruption and TNF transport increase under these conditions, whereas sleep fragmentation has no effect on BBB disruption or transport of TNF in young mice. Previous work has shown that chronic sleep restriction results in wide spread changes in BBB function, including disruption, increased cyclooxygenase-2 levels, and decreases in endothelial and inducible nitric oxide synthase expression, glucose transporter expression, uptake of 2-deoxy-glucose, and tight junction protein expression (He et al., 2014).

These results show that two separate aspects of BBB function, regulation of its integrity and the saturable transport of the cytokine TNF, are affected by CLP and by sleep fragmentation. However, BBB susceptibility shows a clear dichotomy to insult as a function of aging: whereas the aged BBB is dysregulated with sleep fragmentation but resistant to CLP, the young BBB is susceptible to CLP but resistant to sleep fragmentation. These results suggest that the mechanisms by which sleep fragmentation and sepsis lead to alterations in BBB functions are fundamentally different from one another and that a major change in the organism's responses to those insults occurs with aging.

Our results also demonstrate that relatively acute periods of sleep fragmentation can influence interactions between sepsis and BBB integrity and transport of TNF in an age-dependent manner. The protocol we used subjected mice to sleep fragmentation only during the light period, and only for 3 consecutive days. Previous studies using the same device and sleep fragmentation protocol (Ringgold et al., 2013) demonstrate the overall amount of sleep is not greatly affected, yet this acute period of sleep fragmentation is sufficient to alter BBB integrity and TNF transport following sepsis in aged mice. Furthermore, in this protocol there is a 12-h undisturbed sleep opportunity preceding sepsis induction. Our data demonstrate that these periods of recovery from sleep fragmentation do not ameliorate effects of sepsis or sleep fragmentation in aged mice. Lack of effects of sleep fragmentation

on sepsis-induced changes in BBB integrity and TNF transport in young mice may be due to this 12-h recovery period, a possibility we cannot exclude. Under the conditions of this study, there was a tendency for increased BBB TNF transport in young mice subjected to the combination of sleep fragmentation and sepsis relative to values obtained from animals subjected to sepsis alone, but whether this increase would have been greater if sepsis was induced immediately after the last period of sleep fragmentation remains unknown.

In conclusion, we report a differential impact of aging on changes in BBB integrity and TNF transport induced by sleep fragmentation and sepsis. Although additional studies are required, these results suggest that the aged BBB is more sensitive to sleep fragmentation than that of the young. Because aging is associated with disrupted sleep (Ancoli-Israel, 2009; Bliwise, 1993; Edwards et al., 2010) and with increased morbidity and mortality after sepsis, these new results suggest prior sleep history may be an important determinant of clinical outcome in older adults subjected to septic insult.

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Fig. 1.

TNF transport in *young mice* 24 h and 48 h after CLP. Upper panel shows small increases in albumin space that were significant at 48 h post CLP, but not at 24 h. Middle panel shows CLP increased BBB transport of TNF at both time points with no differences between the 24 and 48 h values. Lower panel shows TNF corrected for albumin space. $N = 5$ per group. * $p < 0.05$; ** $p < 0.01$.

Fig. 2.

Effects of CLP on BBB disruption and TNF transport in young and aged mice. Upper left panel shows that 24 h after CLP young mice ($n = 9$) in comparison to young sham mice ($n = 10$) had an increased albumin space, indicative of BBB disruption. Upper right panel shows that CLP did not disrupt the BBB of aged mice ($n = 10$ for sham and $n = 7$ for CLP). Lower left panel shows that in young mice CLP ($n = 5$) increased the rate of TNF transport across the BBB by almost 7-fold in comparison to young sham mice ($n = 9$). Lower right panel shows that in aged mice CLP ($n = 7$) did not affect transport rate comparison to aged sham mice ($n = 10$).

Fig. 3.

Effects of age, CLP, and sleep fragmentation (SF) on BBB disruption and TNF transport. In young mice, CLP and CLP + SF groups had significantly higher brain/serum ratios for albumin (upper left panel) and TNF (lower left panel); the CLP and CLP + SF groups did not differ from each other for either albumin or TNF. In aged mice, SF and CLP + SF groups had significantly higher brain/serum ratios for albumin (upper right panel) and for TNF (lower right panel); the SF and CLP + SF groups did not differ from each other for either albumin or TNF. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to respective sham.