

Peripheral sympathectomy alters neuroinflammatory responses and microglial activity in response to sleep fragmentation in female mice

Noah T. Ashley (✉ noah.ashley@wku.edu)

Western Kentucky University

Ila Mishra

Case Western Reserve University

Keelee B. Pullum

University of Pennsylvania

Kristen N. Eads

Western Kentucky University

Anna R. Strunjas

Western Kentucky University

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Abstract

Background

Sleep loss, either induced by obstructive sleep apnea or other forms of sleep dysfunction, induces an inflammatory response, as commonly measured by increased circulating levels of pro-inflammatory cytokines. Increased catecholamines from sympathetic nervous system (SNS) activation regulates this peripheral inflammation. However, the role that catecholamines play in mediating neuroinflammation from sleep perturbations is undescribed. The aims of this study were to determine (i) the effect of peripheral SNS inhibition upon neuroinflammatory responses to sleep fragmentation (SF) and (ii) whether homeostasis can be restored after 1 week of recovery sleep.

Methods

We measured gene expression levels of pro- and anti-inflammatory cytokines and microglial activity in brain (prefrontal cortex, hippocampus and hypothalamus) of female mice that were subjected to acute SF for 24 hours, chronic SF for 8 weeks, or 7 days of recovery after chronic SF. In each experiment, SF and control mice were peripherally sympathectomized with 6-OHDA (6-hydroxydopamine) or injected with vehicle.

Results

SF elevated cytokine mRNA expression in brain and increased microglial density and cell area in some regions. In addition, chronic SF promoted hyper-ramification in resting microglia upon exposure to chronic, but not acute, SF. Effects of chronic SF were more pronounced than acute SF, and 1 week of recovery was not sufficient to alleviate neuroinflammation. Importantly, 6-OHDA treatment significantly alleviated SF-induced inflammation and microglial responses.

Conclusions

This study provides evidence of SNS regulation of neural inflammation from SF, suggesting a potential role for therapeutics that could mitigate neuroinflammatory responses to sleep dysfunction.

Background

There is accumulating evidence that sleep dysfunction promotes inflammatory responses in brain and peripheral tissues, which can contribute to the development of chronic pathologies, such as metabolic and cardiovascular diseases and neurological disorders [1–3]. The global increase in obesity rates has led to a pronounced rise in the incidence of obstructive sleep apnea [4]. Chronic sleep fragmentation (SF) simulates the interrupted sleep experienced by patients with obstructive sleep apnea and other sleep disorders [5–7]. Concomitantly, sleep loss can increase autonomic sympathetic nervous system (SNS) activation, culminating in the release of norepinephrine (NE) from sympathetic nervous terminals, and NE and epinephrine (E) from the adrenal medullae. These catecholamines are immunomodulatory [3], and

we have recently documented that pharmacological suppression of SNS ameliorates the inflammatory response in peripheral tissues of female mice exposed to acute and chronic sleep dysfunction [7]. However, the role that the SNS plays in mediating neuroinflammatory responses to sleep dysfunction is unexplored.

Sleep loss-induced neurological damage and cognitive decline primarily involves microglia activation in the brain [8]. Microglia cells are resident phagocytes of the brain that act as sentinels of the nervous system to clean debris and regulate inflammatory responses to injury and infection [9]. Sleep deprivation can promote microglia activation in the absence of injury or infection [8], leading to release of pro-inflammatory cytokines and/or increased phagocytosis [10]. Microglia are activated in hippocampus after 5 days of sleep deprivation in rats [9], but acute sleep loss of 8 hours in mice is not sufficient to activate cortical microglia [8]. Thus, the temporal and spatial dynamics of microglia activity during sleep disturbances and deprivation are not fully elucidated.

In the present study we examined the effects of acute and chronic SF on neural inflammatory responses in female mice. Females were chosen because of their general underrepresentation in neurobiological studies [11]. We also assessed whether 7 days of recovery sleep could sufficiently diminish neuroinflammation from chronic SF. To examine the effect of SNS activation on neuroinflammatory responses to SF, we peripherally sympathectomized mice using 6-hydroxydopamine (6-OHDA). We assessed pro-inflammatory cytokine gene expression and microglial morphology in three distinct brain regions (pre-frontal cortex, hippocampus and hypothalamus) that have been explored in previous studies using experimental sleep fragmentation [7,12]. Since peripheral signals of inflammation are proposed to affect neuroinflammation via the neural transmission of vagal afferents reacting to circulating pro-inflammatory agents or the pro-inflammatory agents themselves crossing the blood-brain barrier to directly affect microglia [13], we hypothesized that peripheral sympathectomy would alleviate the neuroinflammatory responses and diminish microglial activity to acute and chronic SF. Finally, we predicted that a 1-week recovery from chronic SF would reverse neuroinflammatory responses as was previously observed when assessing peripheral tissues [7].

Methods

Animals and experimental protocol

Female C57BL/6J mice between 8-12 weeks of age were used. Sleep fragmentation (SF) experiments were performed using automated SF chambers (Lafayette Instrument Company; Lafayette, IN; model 80390) with a thin layer of corn bedding as previously described [7]. These chambers ensure that mice are subjected to sleep fragmentation and not absolute sleep deprivation [12]. Mice were provided *ad libitum* access to water and food and housed in a 12:12-h light-dark photoperiod (lights on- 8am, 21°C ± 1°C) at Western Kentucky University. Subjects were acclimated to the SF chambers for 72 hours prior to the commencement of sleep fragmentation experiments to diminish any carryover effects from the novel cage environment[14]. This study was conducted under the approval of the Institutional Animal Care and

Use Committee at Western Kentucky University (#15-11), and procedures followed the National Institutes of Health's "Guide for the Use and Care of Laboratory Animals" and international ethical standards.

Acute Sleep Fragmentation (Acute SF)

Female mice ($n = 26$) were chemically sympathectomized with a daily subcutaneous injection of 6-OHDA (0.1mg/g body mass/day) for 5 days while the remaining mice ($n = 26$) were injected with the same volume of vehicle (100 μ l of 0.9% NaCl/day). Following the final injection on Day 5, experimental mice ($n = 26$; $n = 13$ injected with vehicle and $n = 13$ injected with 6-OHDA) were subjected to acute SF i.e. a sweeping bar set to move horizontally every 120 seconds for 24 hrs, which simulates the rate of severe sleep apnea among humans ([15]. Controls ($n = 26$; $n = 13$ injected with vehicle and $n = 13$ injected with 6-OHDA) received no sweeping bar movements. 6-OHDA treatment was effective in suppressing SNS activity because serum epinephrine levels were reduced in SF mice receiving 6-OHDA [7].

Chronic Sleep Fragmentation (Chronic SF)

To induce chronic sleep fragmentation, mice ($n = 28$) were subjected to a horizontal sweeping bar set to move every 120 seconds (30 swipes/h) during the light phase (i.e. from 8 am to 8 pm) every day for 8 weeks. To account for increased activity from daily chronic SF, control mice ($n = 26$) were subjected to the same number of bar sweeps as experimental mice for only 3 hours of the light phase (i.e. from 8 am to 11am), albeit at 4 times the rate i.e. 2 swipes/minute, to control for daily activity induced by the bar movement. Mice were subcutaneously injected with either 6-OHDA (0.1mg/g body mass/day; $n = 13$ of SF mice, $n = 13$ of control mice) or vehicle (100 μ l of 0.9% NaCl/day; $n = 15$ of SF mice, $n = 13$ of control mice) between 8-9 am for 5 consecutive days prior to termination of the experiment.

Chronic Sleep Fragmentation + Recovery (Chronic SF + R)

Mice were subjected to the chronic SF protocol above, but post SF, control ($n = 16$) and SF mice ($n = 25$) were subjected to a recovery period (no bar movement) for 7 days during which they were injected with either 6-OHDA (0.1mg/g body mass/day; $n = 13$ of chronic SF + R mice, $n = 8$ of control mice) or vehicle (100 μ l of 0.9% NaCl/day; $n = 12$ of SF + R mice, $n = 8$ of control mice) for 5 days.

For immunohistochemical studies, the experimental protocols mentioned above were followed, except that the chronic SF+ R experiment did not have a separate control group. Hence, in immunohistochemistry experiment 2, there were three sleep paradigms: controls, chronic SF and chronic SF+ R, each with vehicle or 6-OHDA treatment ($n = 5$ per group except vehicle injected chronic SF+ recovery group with $n = 4$).

Gene expression analysis

In all three experiments, 24 hours following the final drug administration, brains of mice ($n = 8/$ group except chronic SF + vehicle group of $n = 10$ mice) were sampled for RTPCR or immunohistochemistry analysis. For measurement of brain gene expression, brains were dissected from decapitated mice and stored in RNAlater solution (Thermo Fischer Scientific) at 4°C until RNA extraction.

RNA was extracted from hippocampus, pre-frontal cortex and hypothalamus using a RNeasy mini kit (Qiagen). RNA concentrations were measured using a NanoDrop 2000 Spectrophotometer (ThermoScientific). Total RNA was reverse transcribed into cDNA using a high-capacity cDNA reverse transcription kit (Life Technologies, Cat number: 4368813). The prepared cDNA was used as template for determining relative cytokine gene expression using an ABI 7300 RTPCR system. Cytokine probes (*IL1 β* , *IL-6*, *TNF α* , *TGF β* ; Applied Biosystems) labelled with fluorescent marker 5-FAM at the 5' end and quencher MGB at the 3' end were used for genes of interest along with 18S (primer-limited VIC-labelled probe) as the endogenous control according to the manufacturer's instructions. Samples were run in duplicate and the fold change in mRNA levels was calculated as the relative mRNA expression levels, $2^{-\Delta\Delta Ct}$ [16]. Briefly, the cycle threshold (Ct) obtained by fluorescence exceeding background levels was used to calculate $\Delta Ct(Ct[\text{target gene}] - Ct[18s])$. Each Ct value was normalized against the lowest Ct value of a control sample. The negative value of this powered to 2 ($2^{-\Delta\Delta Ct}$) was plotted.

Immunohistochemistry

For immunohistochemistry studies, 24 hours following final drug administration, mice were deeply anesthetized using isoflurane vapors and then transcardially perfused ($n = 5/\text{group}$ except recovery + vehicle group of $n = 4$) with ice-cold saline (0.9%) followed by 4% paraformaldehyde prepared in phosphate buffered saline (PBS). Brains were dissected out from perfused mice, post-fixed in the same fixative overnight, cryoprotected in 30% sucrose and stored at -80°C until cryosectioned.

30 μm sections from brains of control ($n = 5/\text{vehicle}$ or 6-OHDA treatment) and acute SF ($n = 5/\text{vehicle}$ or 6-OHDA treatment) mice from the first study, and control ($n = 5/\text{vehicle}$ or 6-OHDA treatment), chronic SF ($n = 5/\text{vehicle}$ or 6-OHDA treatment) and chronic SF+ R ($n = 4$ injected with vehicle, $n = 5$ injected with 6-OHDA) mice from the second study were processed for immunohistochemistry of IBA-1. Sections were rinsed thrice in 10mM PBS, followed by an incubation in blocking solution (2.5% goat serum, 0.1% triton X) for 2 hours and overnight incubation (at 4°C) in the same blocking solution containing anti-Iba-1 (1:300, Catalogue number 019-19741, Wako Pure Chemicals Industries, China). Sections were then incubated with goat anti-rabbit secondary antibody (Alexa fluor 594, catalogue number A11037, Invitrogen, Life Technologies Corporation, Oregon, USA) for 2 hours, followed by washing of sections with 10mM phosphate buffer saline and mounting on slides. After 2 hours of air drying in a dark chamber, slides were mounted and permanently fixed with DAPI+antifade (Vectashield, H-1500, Vector Laboratories, Inc., CA 94010, USA).

Image Analysis

To quantify microglia, every sixth section of the brain was selected and immune-labelled with Iba1 antibody. Iba1 immunoreactivity (-ir) was visualized through a Zeiss Axioplan 2 epifluorescence microscope fitted with a Leica DFC300 FX digital camera. Omission of primary antibodies in control studies confirmed the absence of nonspecific immunoreactivity (data not shown). Iba1-ir cells in captured images were manually counted by two operators blind to the experimental conditions using the cell counting plug-in in Image J (Version 1.45 J; National Institutes of Health, Bethesda, MD, USA). The brightness and contrast of images were adjusted to a firm setting for all sections to aid in visualization of immunostaining. A box of size 80000 μm^2 , 100000 μm^2 and 42000 μm^2 was overlaid on 100x (10x objective, 10x eyepiece) pictures of sections with cingulate cortex (Bregma: +0.74: +0.38), dentate gyrus (Bregma: -1.64: -2.12) and pre-optic area (Bregma: -0.94: -1.28), respectively, and region identification was based on the mouse brain atlas (Paxinos and Franklin, 2001; http://www.mbl.org/atlas170/atlas170_frame.html). Cells were counted in a total of 4 sections. Iba1-ir values were averaged from both sides of bilateral nuclei, and the count from 4 sections was summed for each mice. Total numbers of positive cells per animal were multiplied by six to estimate the number of cells per cingulate cortex, dentate gyrus and pre-optic area.

For morphological analysis of microglia, all analysis were performed on maximum intensity projections (Z-project, maximum intensity function in Image J) of the Z-stacks collected with 0.7 μm increments on a Olympus Flowview (FV1000) confocal microscope with 10X eyepiece and 100X objective (1000X). Microglia were classified as ramified (numerous thin processes, radial branching), primed (thickened processes, increased polarity, and proliferation with reduced secondary branching), reactive (thickened stout processes with highly reduced branching), or amoeboid (rounded soma with no branching; Figure 1A–D) based on standard morphological criteria [17]. Sholl analysis was performed over 20 concentric circles, with the inner radius of 6 μm and outer radius of 35 μm on a total of 15 ramified microglia (5 from cingulate cortex + 5 from dentate gyrus + 5 from pre-optic area) per brain and 5 brain/group. Analysis was done by 3 operators blind to the experimental conditions. Non-overlapping microglia cells that were in a ramified state exhibiting intact microglial processes unobscured by either background labelling or other cells were photographed and analysed.

Statistical Analysis

Data are presented as mean (\pm SE). All statistical analyses were done using GraphPad prism (version 6.0), IBM SPSS Statistics version 20 software as appropriate. Two-way ANOVAs assessed the effect of sleep fragmentation (factor 1), 6-OHDA treatment (factor 2) and their interaction on mRNA expression of cytokines and Iba-1 immunohistochemistry in brain. A three-factor analysis, using Univariate General linear model (GLM) tested the effects of sleep fragmentation (factor 1), 6-OHDA treatment (factor 2), and distance from soma (factor 3), and their interactions on morphology of the Iba-1 staining. Bonferroni multiple comparisons were used for posthoc analysis. $p < 0.05$ was considered statistically significant.

Results

SF-induced effects on cytokine mRNA expression levels in brain

There was a significant effect of acute SF on *IL-1 β* and *IL-6* expression levels in prefrontal cortex (*IL-1 β* : $F_{1,28} = 4.69$, $p = 0.039$, *IL-6*: $F_{1,28} = 8.07$, $p = 0.008$, 2-way ANOVA), while acute SF induced effects on *IL-1 β* expression levels in both prefrontal cortex and hippocampus were dependent on 6-OHDA treatment (prefrontal cortex: interaction- $F_{1,28} = 7.81$, $p = 0.009$, hippocampus: interaction - $F_{1,26} = 6.52$, $p = 0.0169$, 2-way ANOVA). Similarly, in hypothalamus, there was a significant effect of acute SF on *IL-1 β* ($F_{1,28} = 6.07$, $p = 0.020$), and an interaction of acute SF and 6-OHDA on *IL-6* ($F_{1,28} = 16.21$, $p = 0.0004$; 2-way ANOVA). *TNF α* levels were unaffected by the experimental treatment in brain tissues while *TGF β* levels in all three brain regions were significantly affected by acute SF (prefrontal cortex: $F_{1,28} = 8.62$, $p = 0.007$; hippocampus: $F_{1,28} = 14.29$, $p = 0.0008$; hypothalamus: $F_{1,28} = 15.07$, $p = 0.0006$) and 6-OHDA treatment (except in prefrontal cortex; hippocampus: $F_{1,28} = 14.77$, $p = 0.0006$, hypothalamus: $F_{1,28} = 10.55$, $p = 0.0030$; 2-way ANOVA).

There was a significant effect of chronic SF on the expression levels of all genes assessed in prefrontal cortex (except *TNF α* , *IL-1 β* : $F_{1,30} = 4.29$, $p = 0.047$; *IL-6*: $F_{1,29} = 7.512$, $p = 0.0106$; *TGF β* : $F_{1,29} = 6.510$, $p = 0.0163$) along with a significant effect of 6-OHDA treatment and its interaction with chronic SF on *TNF α* and *IL-1 β* , respectively (*TNF α* : 6-OHDA- $F_{1,31} = 4.94$, $p = 0.034$; *IL-1 β* : interaction- $F_{1,30} = 4.498$, $p = 0.042$). Further, hippocampal *TGF β* expression was significantly affected by 6-OHDA ($F_{1,30} = 4.43$, $p = 0.044$) and its interaction with chronic SF ($F_{1,30} = 6.17$, $p = 0.019$; 2-way ANOVA). Similarly, hypothalamic *IL-1 β* expression was significantly affected by 6-OHDA treatment ($F_{1,29} = 19.11$, $p = 0.0002$), and hypothalamic *TNF α* levels were significantly affected by chronic SF ($F_{1,29} = 8.27$, $p = 0.0076$) and 6-OHDA ($F_{1,29} = 28.16$, $p < 0.0001$; 2-way ANOVA).

One week of recovery sleep significantly affected *IL-1 β* expression in hippocampus ($F_{1,28} = 5.97$, $p = 0.021$) and hypothalamus ($F_{1,28} = 5.13$, $p = 0.032$), along with a significant effect of recovery and 6-OHDA treatment on *TNF α* expression in brain (hippocampus: recovery - $F_{1,28} = 51.91$, $p < 0.0001$, 6-OHDA- $F_{1,28} = 6.49$, $p = 0.0166$; hypothalamus: recovery - $F_{1,28} = 4.36$, $p = 0.046$, 6-OHDA- $F_{1,28} = 4.84$, $p = 0.036$). *IL-6* expression levels in prefrontal cortex and hippocampus were significantly affected by recovery (prefrontal cortex: $F_{1,28} = 14.38$, $p = 0.0007$; hippocampus: $F_{1,28} = 7.97$, $p = 0.0087$) and 6-OHDA treatment (prefrontal cortex: $F_{1,28} = 14.98$, $p = 0.0006$; hippocampus: $F_{1,28} = 23.54$, $p < 0.0001$), the expression levels in hypothalamus were significantly affected by 6-OHDA ($F_{1,28} = 42.39$, $p < 0.0001$) and its interaction with recovery ($F_{1,28} = 10.30$, $p = 0.0034$). Neural *TGF β* expression levels were significantly affected by recovery (hippocampus: $F_{1,28} = 11.00$, $p = 0.003$; hypothalamus: $F_{1,28} = 11.37$, $p = 0.002$), 6-OHDA (prefrontal cortex: $F_{1,28} = 5.56$, $p = 0.026$; hippocampus: $F_{1,28} = 12.75$, $p = 0.001$; hypothalamus: $F_{1,28} = 14.48$, $p = 0.0007$) and their interaction (prefrontal cortex: $F_{1,28} = 12.68$, $p = 0.0013$; hypothalamus: $F_{1,28} = 13.81$, $p =$

0.0009), except the effect of recovery and its interaction with 6-OHDA in prefrontal cortex and hippocampus, respectively (2-way ANOVA).

In comparison to controls, *IL-1 β* expression under both SF regimes was significantly increased in brain regions (except hippocampus of chronic SF exp; Bonferroni posthoc test, $p < 0.05$, Fig. 2a, e, i, 3a, i). Acute SF caused a significant reduction in expression levels of *TGF β* in all three brain tissues, and in *IL-6* in prefrontal cortex and hypothalamus (Bonferroni posthoc test, $p < 0.05$, Fig. 2b, d, f, h, j, l), as opposed to chronic SF which lead to a significant increase in *IL-6* expression in prefrontal cortex, *TNF α* in hypothalamus and *TGF β* in hippocampus (Bonferroni posthoc test, $p < 0.05$, Fig. 3b, h, k).

A recovery period of 1 week after chronic SF was enough to negate the effects of chronic SF (except *IL-6* in prefrontal cortex). In fact, *IL-6*, *TNF α* and *TGF β* expression in hippocampus and hypothalamus and *IL-1 β* expression in hypothalamus was significantly lower in recovery mice compared with controls (Bonferroni posthoc comparison, $p < 0.05$, Fig 4f-l). In addition, lack of significant differences between 6-OHDA administered controls and experimental groups in all experiments (except hypothalamic *TNF α* of chronic SF+R experiment), along with significant reduction in hypothalamic *IL-1 β* and *TNF α* in 6-OHDA-treated chronic SF mice, and in *TGF β* in hippocampus and cortex of 6-OHDA treated acute and chronic SF mice, indicated that chemical sympathectomy significantly attenuated SF-induced neuroinflammatory responses (Bonferroni posthoc test, $p < 0.05$, fig. 2-4).

SF-induced effects on microglia activity

Iba-ir cell number was significantly affected by acute SF (cortex: $F_{1,16} = 15.60$, $p = 0.001$; hippocampus: $F_{1,16} = 14.79$, $p = 0.001$; pre-optic area: $F_{1,16} = 6.03$, $p = 0.026$), 6-OHDA treatment (cortex: $F_{1,16} = 15.60$, $p = 0.001$; hippocampus: $F_{1,16} = 16.33$, $p = 0.001$), and their interaction (cortex: $F_{1,16} = 26.49$, $p < 0.0001$; hippocampus: $F_{1,16} = 10.45$, $p = 0.005$; pre-optic area: $F_{1,16} = 5.56$, $p = 0.031$) in all three brain regions, except an effect of 6-OHDA in pre-optic area, as determined by 2-way ANOVA. In all three brain regions, acute SF significantly increased Iba-ir, which was significantly decreased with 6-OHDA treatment (Bonferroni posthoc test, $p < 0.05$, Fig. 5a, e, i). Similarly, Iba-ir soma area was significantly affected by acute SF in pre-optic area ($F_{1,96} = 6.46$, $p = 0.012$), by 6-OHDA treatment in cortex ($F_{1,96} = 12.04$, $p = 0.0008$), and by their interaction in hippocampus ($F_{1,96} = 4.87$, $p = 0.029$) and pre-optic area ($F_{1,96} = 4.07$, $p = 0.047$; 2-way ANOVA), with significantly greater -ir soma area in pre-optic area of mice subjected to acute SF compared with controls (Bonferroni posthoc test, $p < 0.05$, Fig. 5b, f, j). Iba-ir soma area in cortex of acute SF mice was significantly greater than their counterparts pre-treated with 6-OHDA (Bonferroni posthoc test, $p < 0.05$, Fig. 5b).

In the second experiment, there was a significant effect of SF on Iba-ir cell number in cortex ($F_{1,23} = 26.24$, $p < 0.0001$) and hippocampus ($F_{1,23} = 4.43$, $p = 0.023$), of 6-OHDA treatment and its interaction with SF in cortex (6-OHDA: $F_{1,23} = 8.10$, $p = 0.009$; SF x 6-OHDA: $F_{2,23} = 19.30$, $p < 0.0001$; 2-way ANOVA).

Interestingly, Iba-ir cell number in cortex and hippocampus of mice subjected to chronic SF > recovery >

controls (Bonferroni posthoc test, $p < 0.05$ Fig. 6a, e). Furthermore, 6-OHDA treatment significantly reduced Iba-ir in mice subjected to chronic SF (Bonferroni posthoc test, $p < 0.05$; Fig. 6a, e, i). There was a significant effect of SF on Iba-ir soma area in cortex ($F_{2,139} = 6.49, p = 0.002$) and hippocampus ($F_{2,139} = 4.68, p = 0.011$), and of 6-OHDA treatment ($F_{1,139} = 12.51, p = 0.0006$) and its interaction with SF ($F_{2,139} = 6.73, p = 0.002$) on Iba-ir soma area in pre-optic area (2-way ANOVA). Iba-ir soma area was significantly increased in chronic SF mice compared with recovery and controls (except in hippocampus; Bonferroni posthoc test, $p < 0.05$, Fig. 6b, f, j). Further, chronic SF mice had significantly lower Iba-ir soma area in pre-optic area when treated with 6-OHDA (Bonferroni posthoc test, $p < 0.05$, Fig. 6j).

Three-factor univariate general linear models indicated a significant effect of acute SF and 6-OHDA treatment on microglial ramification in ($F_{1,1824} = 19.66, p < 0.0001$) and pre-optic area ($F_{1,1824} = 43.31, p < 0.0001$). Microglia ramification significantly varied with distance from soma (cortex: $F_{18,1824} = 38.38, p < 0.0001$; hippocampus: $F_{18,1824} = 20.97, p < 0.0001$; pre-optic area: $F_{18,1824} = 10.02, p < 0.0001$) and acute SF induced effects were dependent on 6-OHDA treatment in all three regions (acute SF x 6-OHDA interaction - cortex: $F_{1,1824} = 6.124, p = 0.013$; hippocampus: $F_{1,1824} = 25.76, p < 0.0001$; pre-optic area: $F_{1,1824} = 15.99, p < 0.0001$). There was a significant three-way interaction only in pre-optic area (acute SF x 6-OHDA x distance from soma: $F_{18,1824} = 3.38, p < 0.0001$, univariate GLM). In cortex, acute SF significantly increased the ramification at 6.5 μm from the centre of soma when compared with controls (Bonferroni posthoc test, $p < 0.05$, Fig 5c). 6-OHDA significantly affected the microglia ramification in controls, with higher ramification in cortex (between 9 to 16 μm from the soma centre), hippocampus (23 μm from the soma centre) and pre-optic area (at 26 and 29 μm from soma centre; Bonferroni posthoc test, $p < 0.05$, Fig 5c, d, g, h, k, l). Similarly, acute SF mice when injected with 6-OHDA, showed higher ramification in hippocampus at a distance of 29-30.5 μm from the centre of soma (Bonferroni posthoc test, $p < 0.05$, Fig 5g, h).

Chronic SF had more profound effects on microglia morphology with a significant effect of (cortex: $F_{2,2754} = 108.79, p < 0.0001$; hippocampus: $F_{2,2754} = 24.59, p < 0.0001$; pre-optic area: $F_{2,2754} = 72.93, p < 0.0001$), 6-OHDA treatment (cortex: $F_{1,2754} = 5.14, p = 0.023$; hippocampus: $F_{1,2754} = 16.51, p < 0.0001$; pre-optic area: $F_{1,2754} = 113.17, p < 0.0001$), distance from soma (cortex: $F_{18,2754} = 40.82, p < 0.0001$; hippocampus: $F_{18,2754} = 37.55, p < 0.0001$; pre-optic area: $F_{18,2754} = 29.74, p < 0.0001$), and SF x 6-OHDA interaction (cortex: $F_{2,2754} = 52.79, p < 0.0001$; hippocampus: $F_{2,2754} = 37.78, p < 0.0001$; pre-optic area: $F_{2,2754} = 28.68, p < 0.0001$) in all three brain regions, as determined by univariate GLM. In addition, there was a significant interaction effect of distance from soma with SF ($F_{36,2754} = 1.83, p = 0.001$) and 6-OHDA treatment ($F_{18,2754} = 2.65, p < 0.0001$) in cortex and pre-optic area, respectively. There was a significant three-way interaction in hippocampus ($F_{36,2754} = 1.91, p = 0.001$) and pre-optic area ($F_{36,2754} = 2.16, p < 0.0001$), but not in cortex. In comparison with controls, chronic SF caused microglia hyper-ramification in all three brain regions (distance from centre of soma: 14 to 32 μm in cortex, 17 to 26 μm in hippocampus, and 20 to 35 μm in pre-optic area). A significantly higher ramification in cortex at 11 to 32 μm , in hippocampus at 26 to 27.5 and 32 to 33.5 μm , and in pre-optic area at 20 to 35 μm from centre of

soma was observed in chronic SF mice compared with recovery mice (Bonferroni posthoc test, $p < 0.05$, Fig 6c, d, g, h, k, l).

Hippocampal microglial response was still observed after 1 week of recovery, as ramification was significantly higher in mice subjected to recovery at 12.5 to 14 μm distance from centre of soma (Bonferroni posthoc test, $p < 0.05$, Fig 6g, h). Further, 6-OHDA administration diminished the neuroinflammatory response from chronic SF, as evidenced by significantly lower microglia ramification in cortex (at 23 to 33.5 μm distance from centre of soma), hippocampus (at 17 to 23 μm , 26 and 30.5 μm distance from centre of soma) and pre-optic area (at 14, and 20 to 30.5 μm distance from centre of soma) in mice subjected to 6-OHDA treatment following chronic SF, relative to their counterparts injected with vehicle (Bonferroni posthoc test, $p < 0.05$, Fig 6c, d, g, h, k, l). The pre-optic area of recovery mice injected with 6-OHDA had lower microglia ramification at 11 to 14 μm distance from centre of soma, compared with recovery mice not injected with 6-OHDA (Bonferroni posthoc test, $p < 0.05$, Fig 6k, l).

Discussion

Sleep disturbances can promote neuroinflammation and blood-brain barrier disruption mediated by inflammatory molecules produced from astrocytes and microglia. For instance, sleep restriction for 72 h elevated *IL-1 β* , *IL-6* and *TNF α* in hippocampus and basal forebrain of rats [18] and chronic sleep restriction for 21 days elevated *IL-6* and *TNF α* in hippocampus and *TNF α* in prefrontal cortex of rats [19]. Similarly, in the present study, sleep fragmentation (SF) promoted neuroinflammatory responses albeit in a brain region-specific and SF-duration dependent manner. In addition to having proinflammatory roles, elevated *IL-1 β* in brain following acute and chronic SF (except in hippocampus), and *TNF α* in hypothalamus following chronic SF may indicate an accumulation of SRS (sleep regulatory substances) following a period of prolonged wakefulness [20]. The increase in neural *TNF α* response after chronic but not acute SF is in corroboration with previous reports [12,21,22]. An induction of an anti-inflammatory environment in the brain after long-term SF has been reported before [21,23]. Increased hippocampal *TGF β* expression following chronic SF perhaps provided evidence for neuroprotection from inflammation. Further, while primarily pro-inflammatory, IL-6 has been described to play anti-inflammatory roles in some scenarios, causing stimulation of IL-10 synthesis [24] and inhibition of IL-1 and *TNF α* production [25]. While the precise functioning of IL-6 is not deciphered in the present study, opposite trends in its mRNA expression under acute and chronic SF seems to suggest an effort to closely regulate neuroinflammatory response to SF.

Microglial activation is considered a main source of neuroinflammation among other factors [9]. Sleep loss has been shown to affect microglial morphology [26]. In mature CNS, microglia are in a 'resting state' with highly ramified and motile processes, continuously surveying the surrounding environment. In response to injury, microglia shift from resting to a primed phagocytic 'activated' state [26]. Sleep loss, in the absence of injury, has been reported to increase phagocytic activity of microglia [8]. On the other hand, psychological and mechanical stress has also been shown to increase the microglial secondary branching in the cortex of rats [27,28], thus suggesting that microglial activation in response to

homeostatic perturbations can range from mild non-inflammatory hyper-ramified phenotype to pro-inflammatory, apoptotic and phagocytic phenotypes [26]. Unlike chronic OSA and SF, neither OSA nor sleep deprivation/restriction of 1 night has been previously reported to increase the phagocytic activity of microglia [8,29]. Therefore, we surmise that perhaps acute SF of 1 night might affect the mild 'non-inflammatory' instead of the 'activated' phagocytic morphology of microglia. Absence of microglial hyper-ramification in acute SF mice suggested that SF-induced neuroinflammatory changes may not necessarily be associated with changes in microglial morphology [26]. On the other hand, increased ramification after chronic SF suggests that chronic sleep perturbations induced microglial morphological changes that may include an increase in 'activated' phenotype involved in enhanced phagocytosis of synaptic elements [8] and an increased ramification of the 'resting' phenotype possibly involved in increased surveillance and scanning of microenvironment [27]. Given the reversibility and complexity of microglia morphology, especially across brain regions [30], we analysed only the ramified morphology of microglia. SF-induced alterations in the phagocytic phenotype need to be tested in future studies, and thus presently cannot be ruled out.

Usually, inflammation from sleep loss return to basal levels after sleep recovery. However, depending on duration of sleep loss and recovery, some immune components may remain altered after sleep recovery [31]. In this study, while seven days of recovery completely alleviates inflammatory responses in peripheral tissues from a previous study [7], it was not sufficient to restore homeostasis in brain. Elevated Iba-ir in all three brain regions and increased *IL-6* expression in cortex of recovery mice indicated that neuroinflammatory responses persisted at least 1 week following chronic SF. Consistent with our finding, a recent study reported that even after three weeks of recovery from sleep loss, neuronal apoptosis, microglial activation and IL-6 response still occurred in hippocampus of mice [32]. Furthermore, similar to our results, anti-inflammatory responses lower than baseline levels have been reported in mice subjected to 20 days of recovery post SD of 192 hours [21]. In addition, we documented a significant reduction in cytokine gene expression in brain after 7 days of recovery sleep. Perhaps, these changes may be a compensatory response that limits neurological and cardiovascular dysfunction related to SF-induced hyperactivation of inflammation over prolonged periods of SF. Irrespective of direction of deviation from baseline, it is plausible that one possible mediator of delayed return of neuroinflammatory to baseline homeostatic state was SNS and not the hypothalamic-pituitary-adrenal axis, since high levels of NE were found after 1 week of recovery sleep [7].

Our initial hypothesis that the SNS contributes to neuroinflammatory responses to sleep loss has been supported, as chemical sympathectomy significantly altered the inflammatory responses in all three SF paradigms examined. These results reiterate the complexities of potential NEI (neuro-endocrine-immune) interactions by which SF can affect immune functioning [31,33]. Peripheral sympathectomy also successfully alleviated SF-induced neuroinflammatory responses. Perhaps one or more of the following established mechanisms contributed to these results: (1) a passive diffusion of peripheral cytokines to brain via circumventricular organs or an active transport with carrier proteins across blood brain barrier (BBB) affected the microglial activity and neuroinflammatory processes (2) peripheral signals stimulated

the endothelial cells of BBB to secrete molecules affecting activity of neurons and glia and/or (3) the autonomic nervous system itself via vagal afferents activated the neuroinflammatory responses [10].

Interestingly, 6-OHDA treatment also altered baseline cytokine levels. More specifically, *TGFβ* in hippocampus and *IL-6* in hypothalamus was significantly lowered, while *IL-1β* in hippocampus was significantly increased in control mice after chemical sympathectomy in acute SF experiment. *IL-6*, *TNFα* and *TGFβ* in hypothalamus and hippocampus (except *TNFα*) of control mice were significantly attenuated after 6-OHDA treatment in the recovery experiment. The effects of chemical sympathectomy on baseline immune function in chronic SF experiment were less profound with only a significant reduction in hypothalamic *TNFα* expression in controls treated with 6-OHDA. Such differences between the three experiments could be attributed to the differences in experimental paradigms and duration of treatments involved (cf. Figure 1). Lastly, our data suggests that baseline neuroinflammatory responses of hypothalamus and hippocampus were more susceptible to peripheral 6-OHDA treatment than the cortex. Further studies are warranted to understand how peripheral signals activate neuroinflammation across different brain regions.

Historically, the bias towards use of male rodents in research has resulted in data gaps to effectively discern the effects of sleep abnormalities upon female physiology. Given the sexual differences in susceptibility to diseases including neurological and immune diseases [34,35], this study provides critical insight regarding effects of sleep disruption on female peripheral and neural immune responses. Sex steroids regulate the development and maturation of immune cells and responses [35]. As result, inflammatory profiles [34,36] and microglial activity [36] differ between the sexes. Therefore, caution is warranted before extrapolating these results to responses in males. Paradoxical SD affects estrous cyclicity in rats [37]. Since we the reproductive status was not tested in mice of our study, the involvement of reproductive hormones in regulating SF-induced inflammation and microglial responses cannot be ruled out. Restraint stress also affects phasing of the estrous cycle in female rats, although there is large individual variation [38]. Because sample sizes in our study were small, future studies should evaluate the effect of estrous cycling upon SF-induced inflammation using a greater sample size.

Conclusion

This study systematically evaluated the SF-associated neuroinflammation and microglial responses of female mice. Changes in inflammatory responses seemed to be reflective of stress-axes activation and were related to the duration of SF [7]. Unlike previous reports, our experimental approach of exposing mice to 8 weeks of SF can more accurately be expected to mimic sleep abnormalities, such as OSA and the diseases associated with it, in the modern world. Moreover, it is important to emphasize that a recovery sleep of seven nights following such chronic sleep fragmentation was clearly insufficient for a full return to homeostasis. Importantly, to our knowledge this is the first study showing chronic SF-induced subtle changes in the morphology of microglia primarily characterised as 'resting'. Finally, this study provides evidence of a critical contribution of SNS in development of an inflammatory state. These

findings could lead to novel therapeutic interventions that target the SNS for treating inflammation-dependent disorders, such as cardiovascular diseases.

Declarations

Availability of data and materials

The data generated for this study are available upon request to the corresponding author.

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Author information

Affiliations

Department of Biology, Western Kentucky University, KTH3007, 1906 College Heights Blvd #11080, Bowling Green, KY, 42101-1800, USA

Ila Mishra, Keelee B. Pullum, Kristen Eads, Anna R. Strunjas, Noah T. Ashley

Harrington Discovery Institute, Case Western University, 11407 Euclid Ave, Cleveland, OH, 44106, USA

Ila Mishra

Department of Biology, University of Pennsylvania, 433 S University Ave, Philadelphia, PA, 19104, USA

Keelee B. Pullum

Contributions

IM wrote the primary draft, performed the experiments and primary analysis, KBP, KE and AS analysed the data, and NTA designed the study, wrote the final draft and acquired funding. All authors read and

approved the final manuscript.

Ethics declarations

All animal studies were conducted under the approval of the Institutional Animal Care and Use Committee at Western Kentucky University (#15-11), and procedures followed the National Institutes of Health's "Guide for the Use and Care of Laboratory Animals" and international ethical standards.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

Experimental Protocol

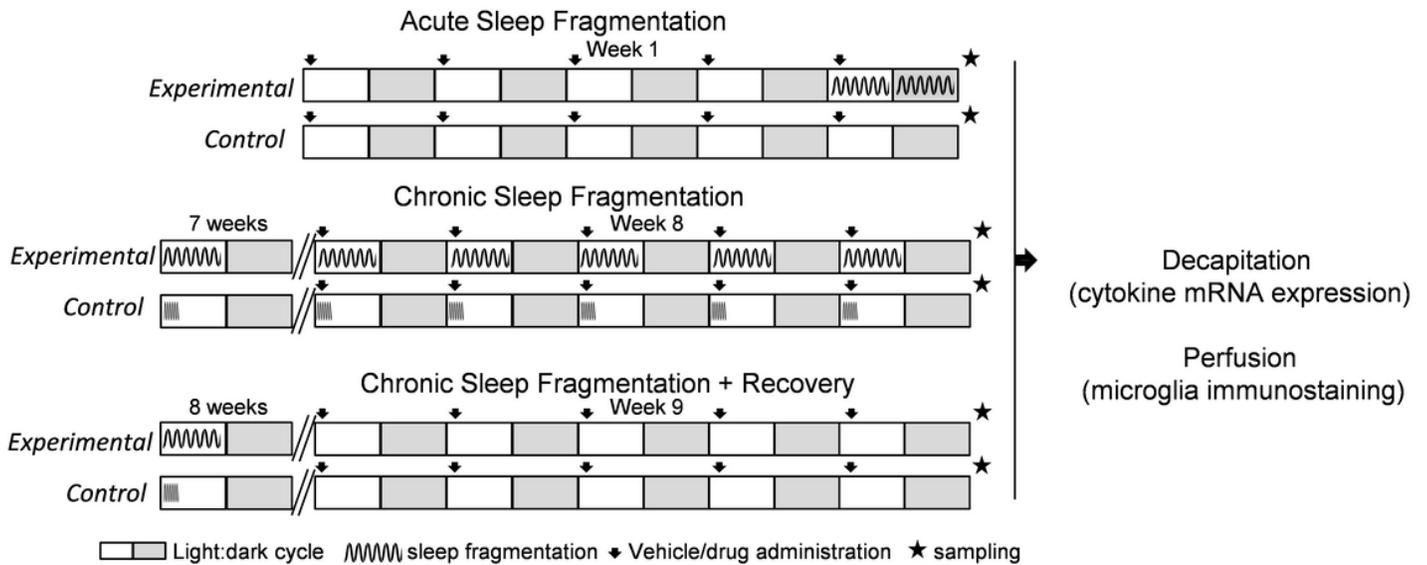


Figure 1

Experimental protocol. Three experiments were performed with experimental mice subjected to sleep fragmentation (SF) in an automated sleep fragmentation chamber for 24 hr (experiment 1: Acute SF), 8 weeks (experiment 2: Chronic SF), or 8 weeks of chronic SF followed by undisturbed sleep of 1 week (experiment 3: Chronic SF + recovery). A horizontally moving automated sweeping bar set to swipe across the chamber every 2 minutes ensured experimental group mice were sleep fragmented. Controls in chronic SF and recovery experiment were also subjected to a sweeping bar, although at a higher speed, and for lesser duration (2 swipes/minute for 3 hours aligned with lights on) to control for the increased activity induced effects between groups. During the recovery phase, mice of both- control and experimental groups were not subjected to sweeping bar swipes. On last 5 days of the experiments, mice were injected subcutaneously with 0.9% saline or 6-OHDA (6-hydroxydopamine). After 24 hours of final injection, mice were either decapitated for tissue gene expression study or transcardially perfused for immunohistochemical study. All mice were > 8 weeks of age and were subjected to 12h light: 12h dark cycles with lights on at 8am and Lights off at 8pm, and provided food and water *ad libitum*.

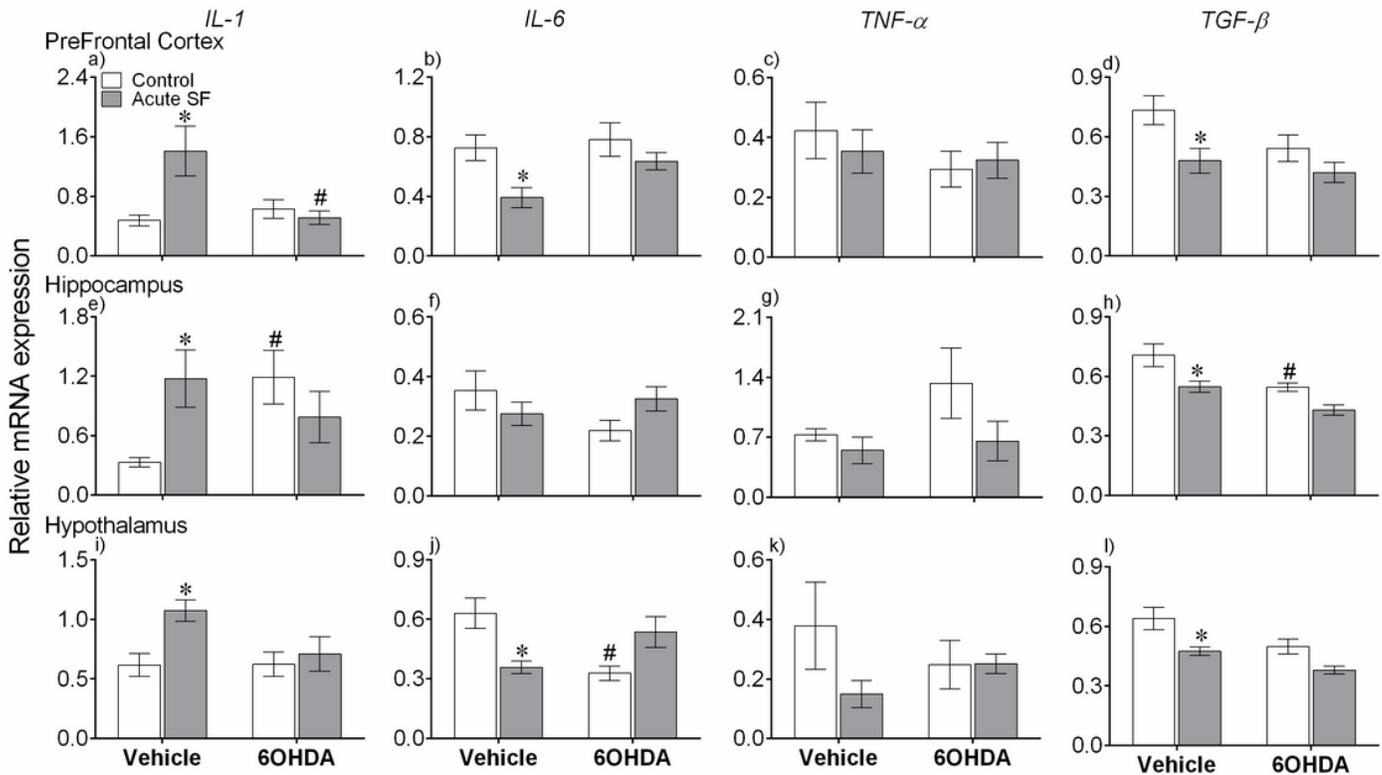


Figure 2

Acute Sleep fragmentation altered cytokine mRNA expression levels in brain tissues. Mean (\pm SE) gene expression levels of *IL-1*, *IL-6*, *TNF α* , and *TGF β* in Prefrontal cortex (a-d), hippocampus (e-h), and hypothalamus (i-l) of vehicle-injected or chemically sympathectomized mice subjected to control or acute SF (n = 8/group). Asterisk (*) and pound (#) indicate a significant effect of SF and chemical sympathectomy, respectively, as determined by Bonferroni posthoc test followed by 2-way ANOVA. For statistical significance, alpha (α) was set at 0.05.

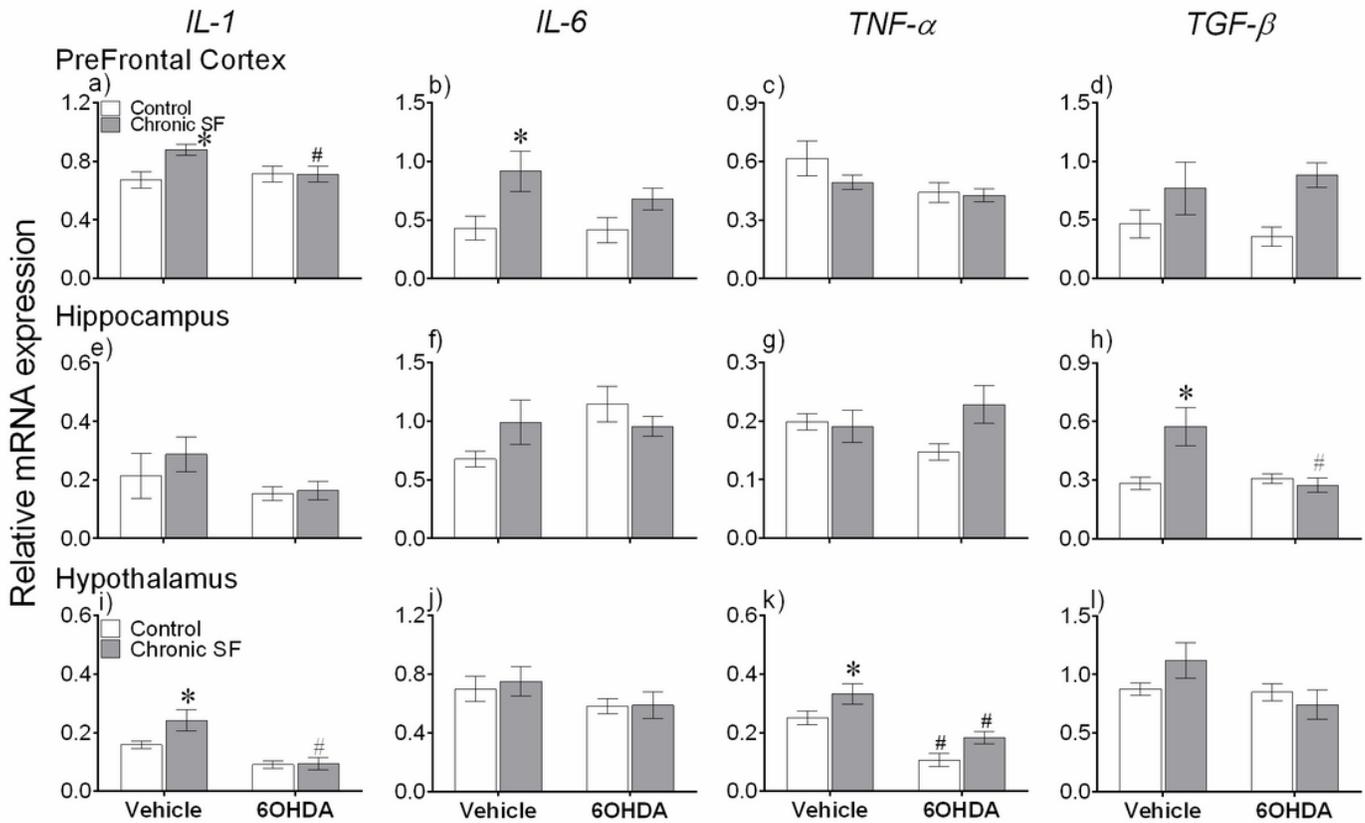


Figure 3

Chronic Sleep fragmentation altered cytokine mRNA expression levels in brain tissues. Mean (\pm SE) gene expression levels of *IL-1*, *IL-6*, *TNF α* , and *TGF β* in Prefrontal cortex (a-d), hippocampus (e-h), and hypothalamus (i-l) of vehicle-injected or chemically sympathectomized mice subjected to control or chronic SF ($n = 8$ /group except for chronic SF + vehicle group of $n = 10$ mice). Asterisk (*) and pound (#) indicate a significant effect of SF and chemical sympathectomy, respectively, as determined by Bonferroni posthoc test followed by 2-way ANOVA. For statistical significance, alpha (α) was set at 0.05.

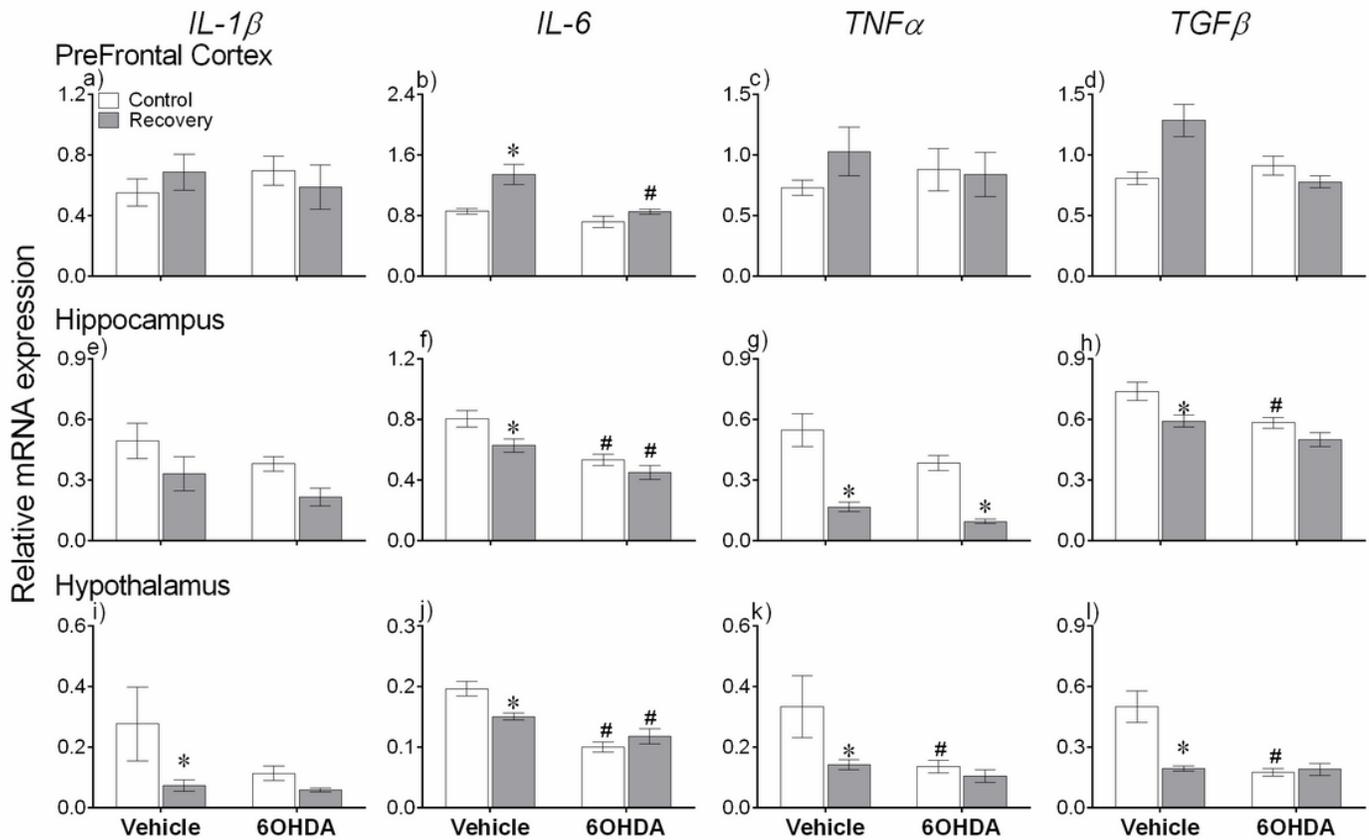


Figure 4

One week of recovery altered cytokine mRNA expression levels in brain tissues. Mean (\pm SE) gene expression levels of *IL-1*, *IL-6*, *TNFα*, and *TGFβ* in Prefrontal cortex (a-d), hippocampus (e-h), and hypothalamus (i-l) of vehicle-injected or chemically sympathectomized mice subjected to control or one week recovery following chronic SF (n = 8/group). Asterisk (*) and pound (#) indicate a significant effect of SF and chemical sympathectomy, respectively, as determined by Bonferroni posthoc test followed by 2-way ANOVA. For statistical significance, alpha (α) was set at 0.05.

Figure 11

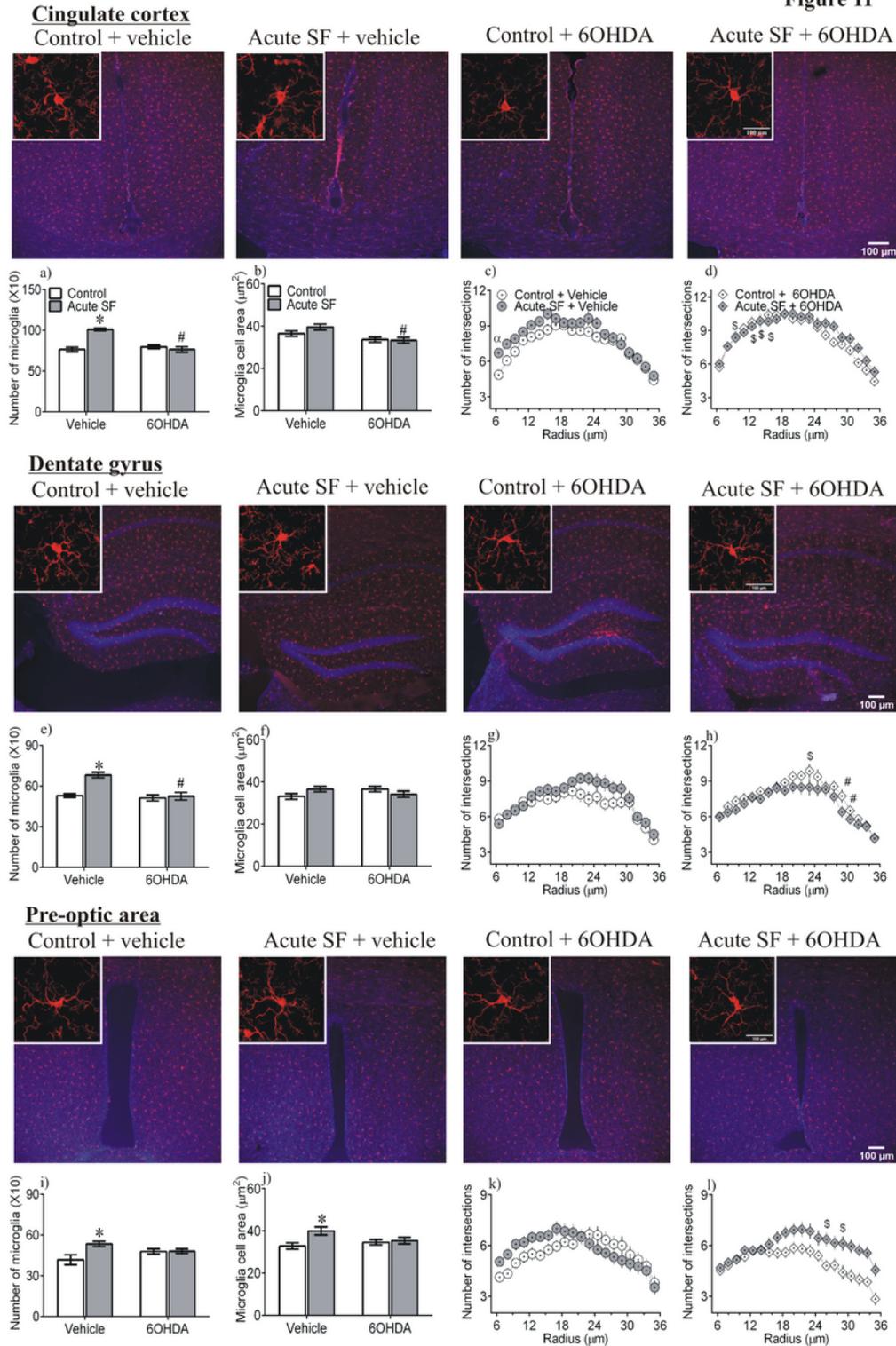


Figure 5

Acute sleep fragmentation altered microglia activation. Mean (± SE) Iba-1-ir cell number (a, e, i), cell area (b, f, j), and ramification as determined by sholl analysis (c-d, g-h, k-l) in cortex (cingulate cortex; upper panel), hippocampus (dentate gyrus; middle panel) and hypothalamus (pre-optic area; lower panel) of vehicle-injected or chemically sympathectomized mice subjected to control or acute SF (n = 5/group). The magnified view in each micrograph represents ramified microglia (magnification: 1000x) subjected to

shall analysis. Asterisk (*) and pound (#) indicate a significant effect of SF and chemical sympathectomy, respectively, on Iba-1-ir cell number and area, as determined by Bonferroni posthoc test followed by 2-way ANOVA. α and # indicate a significant difference of acute SF + vehicle from control + vehicle and acute SF + 6-OHDA, respectively, while \$ indicates a significant difference between the control mice subjected to vehicle or 6-OHDA treatment, as determined by Bonferroni posthoc test following general linear model analysis of microglia ramification. For statistical significance, alpha (α) was set at 0.05.

Figure 12

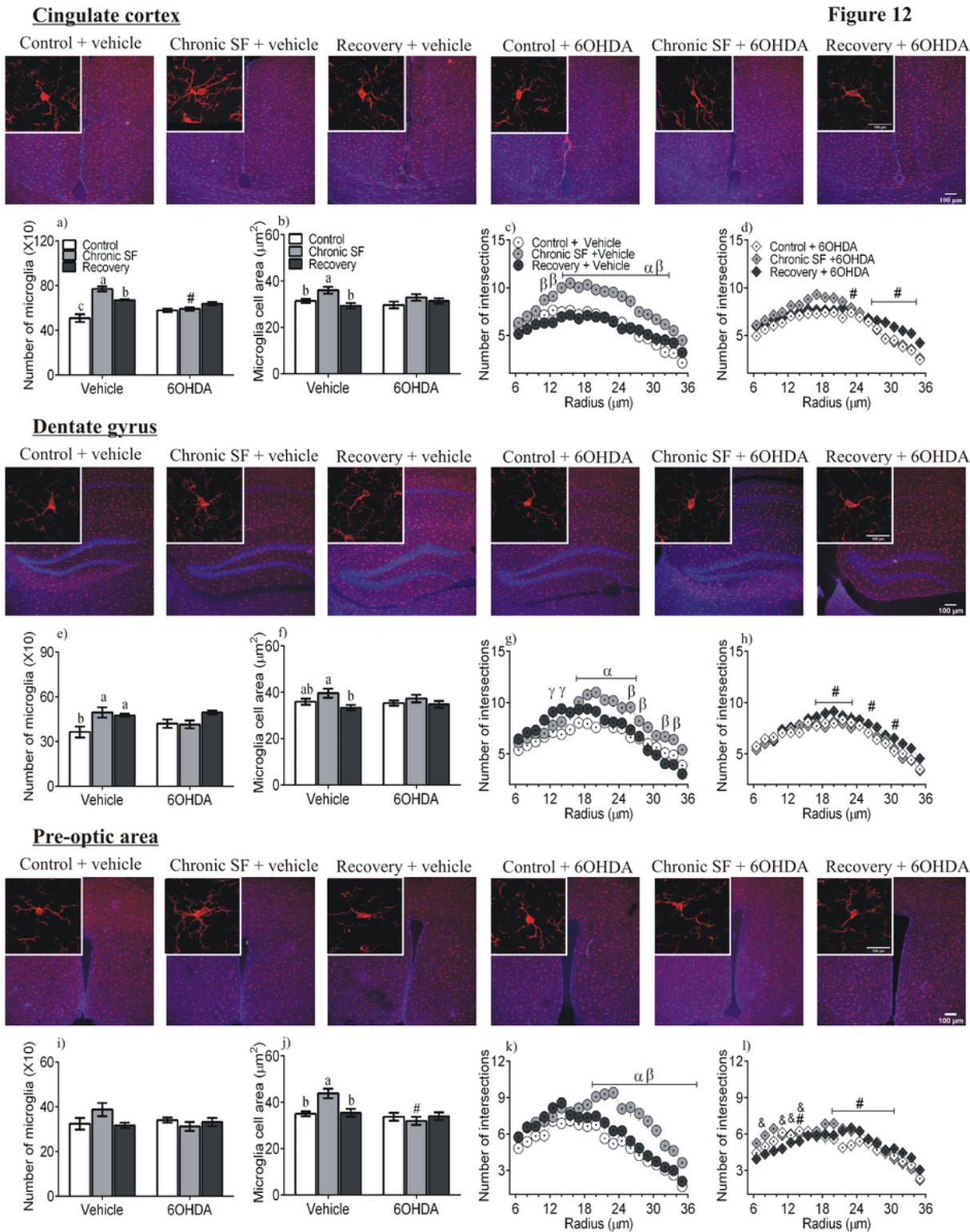


Figure 6

Chronic Sleep fragmentation and one week of recovery altered microglia activation. Mean (\pm SE) Iba-1-ir cell number (a, e, i), cell area (b, f, j), and ramification as determined by sholl analysis (c-d, g-h, k-l) in cortex (cingulated cortex; upper panel), hippocampus (dentate gyrus; middle panel) and hypothalamus (pre-optic area; lower panel) of vehicle-injected or chemically sympathectomized mice subjected to control, chronic SF, or a recovery of 1 week following chronic SF (n = 5/group except vehicle treated

chronic SF + recovery group of n = 4 mice). The magnified view in each micrograph represents the ramified microglia (magnification: 1000x) subjected to sholl analysis. Asterisk (*) and pound (#) indicate a significant effect of SF and chemical sympathectomy, respectively, on Iba-1-ir cell number and area, as determined by Bonferroni posthoc test followed by 2-way ANOVA. General linear model followed by Bonferroni posthoc test tested the effects on microglia ramification. α and β indicate a significant difference of acute SF from control and recovery mice, respectively, while γ indicates a significant difference between recovery and control mice. Drug-induced effects are indicated by # and &, presenting a significant difference between vehicle and 6-OHDA treated mice of chronic SF and recovery, respectively. For statistical significance, alpha (α) was set at 0.05.