Sleep Fragmentation Induces Cognitive Deficits Via NADPH Oxidase-Dependent Pathways In Mouse

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ONLINE DATA SUPPLEMENT

Supporting information for Online Supplement:

Methods

Animals: Male hemizygous *gp91phox-*$^{/Y}$ (B6.129S-*Cybb*^{tm1Din}/J) mice (20-22 grams) and C57BL/6J mice (20-22 grams) were purchased from Jackson Laboratories (Bar Harbor, Maine), housed in a 12 hr light/dark cycle (lights on from 7:00 am to 7:00 pm) at a constant temperature (26 ±1^{o}C). Mice were housed in groups of four in standard clear polycarbonate cages, and were allowed access to food and water *ad libitum*. All behavioral experiments were performed during the light period (between 9:00 am and 12:30 pm). Mice were randomly assigned to either sleep control (SC) or sleep fragmentation (SF) exposures. The experimental protocols were approved by the Institutional Animal Use and Care Committee and are in close agreement with the National Institutes of Health *Guide in the Care and Use of*. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Sleep Fragmentation Exposures: Animals were maintained in custom made cages operated under a 12 hour light-dark cycle (7:00 am-7:00 pm) for 14 days prior to behavioral testing, and then for the duration of behavioral testing. The custom fabricated device used to induce SF in mice has been previously described (1) (Lafayette Instruments, catalog # Model 80390, Lafayette, IN). SF was performed by switching on the sweeper to a timer mode in the cage. In this mode, the sweeper requires around 9 sec to sweep the floor of the cage one way. When it reaches the end of the cage, a relay engages the timer which pauses for 2 min before enabling the sweeper to move in the

opposite direction. Between the 2 intervals, the animal remained undisturbed. During sweeper motion, animals would need to step over the sweeper, and then continue with their unrestrained behavior. If the mouse was asleep, a brief tactile stimulation elicits a brief arousal by the sweeper motion. This method prevents the need for human contact and intervention, and minimizes physical activity during the entire sleep disruption procedure. Since on average, 30 episodes of arousal per hour occur in patients with moderate to severe sleep apnea (i.e., every 2 min), our aim was to mimic closely the severe disease condition, and thus, chose the interval of 2 min for the SF paradigm.

Surgical procedure and implantation of telemetric transmitter and electrodes: All surgical procedures were performed under sterile conditions and general anesthesia (Induction: 3% isoflurane and 1 liter per min of O_2. Maintenance: 2% isoflurane and ½ liter per min of O_2). First, the animals were positioned in sternal recumbency, and a dorsal neck incision of 2-3 cm was made through the skin along the dorsal midline, covered with a sterile bandage, after which, a 1.5 - 2 cm incision was performed through the skin and abdominal wall along the ventral midline. A telemetric transmitter weighing 3.5 g, F20-EET (DSI, Minnesota, USA), which allows simultaneous monitoring of two biopotential channels, temperature and locomotor activity was inserted, biopotential leads were exteriorized, and the abdominal wall was closed using 4-0 non-absorbable suture with a simple interrupted pattern. The 2 pairs of biopotential leads were then advanced subcutaneously from the ventral abdomen incision to the dorsal neck incision using a trocar. Animals were then fixed in a stereotaxic apparatus for implantation of EEG electrodes, with the first pair of biopotential leads being fixed to the skull above the

frontal area (1mm anterior to bregma and 2mm lateral to mid sagittal suture for one of the leads, and 1mm anterior to lambda and 2.5 mm lateral to mid sagittal suture for the other lead). The other pair of biopotential leads was placed within the same bundle of dorsal neck muscles for the recording of nuchal EMG.

Acclimatization, sleep recording and sleep fragmentation: After complete recovery from surgery, mice were transferred to the SF cages for habituation to the cage and the sweeper. The recording cages were placed on a DSI telemetry receiver (RPC-1), which was in turn connected to an acquisition computer through a data exchange matrix. After at least one week of acclimatization in the cages, the magnetic switch of the transmitter was activated, and polygraphic recordings were begun at 7.00 am. Physiological data were continuously acquired for 24h using Dataquest ART acquisition software (DSI, Minnesota, USA; version 3.1), at a sampling rate of 500 Hz. First, baseline (BL) data was acquired for 24h. Then the mice were subjected to SF for 14 days from (7am – 7pm). Following SF exposure, baseline data and 14th day data were first scored automatically using Sleepsign software (Kissei Comtec, Japan) (1-2)) , and records were visually confirmed or corrected as needed by an investigator who was blinded to the experimental condition.

Behavior was classified into 3 different states: wake, slow wave sleep (SWS) and rapid eye movement (REM) sleep. EEG during W had low-amplitude, high-frequency (desynchronized) waves. During wake, EMG records showed gross body movement artifacts and behaviorally, animals had grooming, scratching and orienting activity. The SWS stage was characterized by low-frequency, high-amplitude (synchronized) EEG with a considerable reduction in EMG amplitude. The mice assumed a curled recumbent

posture during this period. REM sleep was characterized by desynchronized EEG, and a drastic reduction in EMG (muscle atonia). Sleep-related low frequency (delta) activity was also derived from the records using bandpass filtering of 1– 4.0 Hz. Delta power was computed by using SleepSign software by Fast Fourier Transform (FFT), which was based on 512 points corresponding to 10 sec epochs, at a sampling rate of 250 Hz with Hanning as the window filter of FFT. Those SWS epoch which showed movement artifacts were excluded when computing delta power, since EEG signals are especially sensitive to movement, with the resulting artifact specifically enhancing signals in the delta band.

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Behavioral Testing: The Morris water maze was used to assess spatial reference learning and memory, as well as working memory. The maze protocol is similar to that described by Morris (3) with modifications for mice. The maze consisted of a white circular pool, 1.4m in diameter and 0.6m in height, filled to a level of 35cm with water maintained at a temperature of 21ºC (4). Pool water was made opaque by addition of 150 ml of non-toxic white tempera paint. A Plexiglas escape platform (10 cm in diameter) was positioned 1 cm below the water surface and placed at various locations throughout the pool. Extramaze cues surrounding the maze were located at fixed locations, and

visible to the mice while in the maze. Maze performance was recorded by a video camera suspended above the maze and interfaced with a video tracking system (HVS Imaging, Hampton, UK).

Briefly, a standard place-training reference memory task was conducted on mice in the water maze following exposure to 14 days of SF or SC. One day prior to place learning, mice were habituated to the water maze during a free swim. Place learning was then assessed over six consecutive days using a spaced training regimen that has been demonstrated to produce optimal learning in mice (5). Each training session consisted of three trials separated by a 10 minute inter-trial interval (ITI). On a given daily session, each mouse was placed into the pool from 1 of 4 quasirandom start points (N, S, E or W) and allowed a maximum of 90 seconds to escape to the platform where the mice were allowed to stay for 15 sec. Mice that failed to escape were led to the platform. The position of the platform remained constant during the trials. 24 h following the final training session, the platform was removed for a probe trial to obtain measures of spatial bias. To assess the performance in the water maze, mean escape latencies and swim distance were analyzed.

Reference memory: Retention tests were carried out 14 days after acquisition of the task. In the retention test, performance in a single session (two trials) was assessed, and the mean average performance of the two trials was calculated.

Elevated plus maze (EPM): The elevated plus maze (EPM) was used to assess anxiety. The basic measure is the animal preference for dark, enclosed places over bright, exposed places (6-7). A 60 w light was placed above the apparatus and the test was video taped by an overhead camera. Mice were placed in the center of the maze facing a closed arm, and

allowed to explore for 10 min in isolation. Each mouse received one videotaped trial. Mice prefer to enter into closed arms compared to open arms. Time spent in the dark area is viewed as avoidance or anxiety-like behavior. The following parameters were scored: (a) Percent time spent in open and closed arms; (b) number of entries to closed arms; (c) Time spent in the center. An arm entry was defined as the entry of all four feet into either one of the closed arm. Of note, the maze was cleaned with 30 % ethanol between trials to remove any odor cues.

Forced swimming test (FST): Briefly, mice were individually forced to swim in an open cylindrical container (diameter 14 cm, height 20 cm), with a depth of 15 cm of water at 25 ± 1 °C. The immobility time, defined as the absence of escape-oriented behaviors, was scored during 6 min, as previously described (7-9). Each mouse was judged to be immobile when it ceased struggling, and remained floating motionless in the water, making only those movements necessary to keep its head above water. The average percentage immobility was calculated by a blinded experimenter.

Measurement of ATP levels

Snap-frozen cortical tissues were pulverized on liquid nitrogen and nuclear acids were extracted with ice-cold 5% trichloroacetic acid (TCA). The mixture was sonicated with a probe sonicator for 10 pulses and centrifuged at 10,000 g and 4°C for 15 min. The supernatant was collected and neutralized with Tris buffer (1 M, pH 7.8). ATP levels were then measured using the ATP Bioluminescence Assay Kit CLS II (Roche Applied Science, Indianapolis, IN). Protein concentrations in the supernatant were determined using the Bio-Rad Dc protein assay kit (Bio-Rad, Hercules, CA) to normalize ATP levels.

Assessment of AMP Kinase α Activation

Snap-frozen cortical tissues were pulverized on LN_2 and immediately homogenized in 1% sodium dodecyl sulfate (SDS) preheated to 92°C. The homogenate was centrifuged at 14,000 g for 15 min and the supernatant containing total cellular proteins was collected. This method was previously shown to effectively inhibit activation of phosphorylation and dephosphorylation processes associated with many routine protein preparation protocols (10-11)). The Pierce Microplate BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL) was used to determine the protein concentration and samples were then subjected to Western blotting analysis for AMPK activation. The same blot was used for detection of the phospho-AMPK-α (anti-pAMPKα, Cell Signaling Technology, Danvers, MA) and the total AMPKα (anti-AMPKα, Cell Signaling Technology) and positive signals were visualized with enhanced chemiluminescence (ECL).

NADPH Oxidase Expression:

qRT-PCR analysis of p47phox and p67phox were performed using ABI PRISM 7500 System (Applied Biosystems, Foster City, CA). RNA from frontal cortex in SF and control mice was prepared with Qiazol (Qiagen,Valencia,CA) . cDNA synthesis was performed using a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). B-actin was used as a reference gene to normalize the expression ratios for the gene of interest. Taqman primer and probes for p47phox and p67phox were purchased from Applied Biosystems assays number Mm00447921_m1 and Mm00726636_m1 respectively. One microgram of total RNA was used to generate cDNA templates and TaqMan® Master Mix Reagent Kit (Applied Biosystems, Foster City, CA) was used to amplify and quantify the p47phox and p67phox transcript in 20 μl reactions. Duplicate PCR reactions were performed in 96-well in parallel with the B-actin rRNA. The steps

involved in the reaction program included: the initial step of 2 minutes at 50^{o}C; denaturation at 95^{o}C for 10 min, followed by 45 thermal cycles of denaturation (15 seconds at 95^{o}C) and elongation (1 min at 60^{o}C). Expression values were obtained from the cycle number (Ct value) using the Biosystems analysis software. These Ct values were averaged and the difference between the B-actin Ct (Avg) and the gene of interest Ct (Avg) was calculated (Ct-diff). The relative expression of p47phox and p67phox were analyzed using the $2^{-\Delta\Delta CT}$ method.

NADPH oxidase activity: NADPH oxidase activity was assessed by measuring NADPH-dependent superoxide production as described (12) with minor modification. Briefly, cortical tissue homogenate (10 μg) was incubated in 200 μl of assay buffer containing acetylated cytochrome c (100 μM) in a 96-well plate at 30°C. NADPH (200 μM) was then added in the absence or presence of superoxide dismutase (SOD, 3 U/μl) and the reduction of cytochrome c was monitored at 550 nm for 10 min. NADPH oxidase activity was calculated as the SOD-inhibitable reduction of cytochrome c. All chemicals were from Sigma (St. Louis, MO).

Lipid Peroxidation and 8-OHDG Assay: MDA-586 kits (OxisResearch, Portland OR) were used to measure the relative malondialdehyde (MDA) production , a commonly used indicator of lipid peroxidation (50), in frontal brain cortex according to the manufacturer's instructions. Briefly, after anesthesia with pentobarbital (50 mg/kg intraperitoneally), mice were perfused with 0.9% saline buffer for 5 minutes and the cortex was dissected, snap frozen in liquid nitrogen, and stored at –80°C until assay the following day. Cortical tissues were homogenized in 20 mM phosphate buffer (pH 7.4) containing 0.5 mM butylated hydroxytoluene to prevent sample oxidation. After protein

concentration measurements, equal amounts of proteins (2.0–2.5 mg protein from each sample) were used in triplicate to react with chromogenic reagents at 45°C in 500 µL buffer for 1 to 2 hours. The samples were then centrifuged and clear supernatants measured at 586 nm. The level of MDA production was then calculated with the standard curve obtained from the kit according to the manufacturer's instructions.

8-hydroxydeoxyguanosine (8-OHDG): Levels of 8-OHDG were measured in frontal brain cortex using a commercially available assay (Cell Biolabs, San Diego, CA). Briefly, cortical samples or 8-OHDG standards were first added to an 8-OHDG/BSA conjugate preabsorbed enzyme immunoassay plate. After a brief incubation, an anti–8-OHDG mAb was added, followed by an horseradish peroxidase-conjugated secondary antibody. The 8-OHDG content in the cortical samples was then determined by comparison with the 8-OHDG standard curve.

Data Analysis: To elucidate the nature of identified interactions between control baseline sleep characteristics and those emerging after 15 days of SF in WT and *gp91phox*$^{-/Y}$, the data were analyzed by one way repeated measure ANOVA. First, overall statistical significance was determined for the 24-h period between the treatment groups (baseline and SF), followed by post-hoc Tukey tests, as needed. Delta power during SWS, wake episodes and the latency of SWS after each episode of wake were also treated with similar statistical approaches. Similar statistical approaches were used to compare the entire training period between the treatment groups. In addition, two-way repeated measures ANOVA were used to analyze each trial blocks, followed by post-hoc Tukey tests. The same statistical approaches were used to compare probe trial, reference memory, EPM and FST. For all comparisons, a p value <0.05 was considered to achieve

statistical significance. For behavioral test assessments, all the experimental conditions, the data were divided into 6 blocks (containing 3 trials/day). We used a multivariate MANOVA model (SPSS software 11; Chicago) that included latency, pathlength and swim speed and Two between factors: (1) Groups (four levels): SC C57BL6J, SF C57BL6J , SC *gp91phox*$^{-/Y}$ and SF *gp91phox*$^{-/Y}$ (2) Condition (two levels): SC or SF. All *F* statistics are reported using Pillai's Trace. The interaction of three different factors, i.e., time, condition and group were determined using this mixed model repeated measures MANOVA. For the biochemical assays of MDA and 8-OHDG and NADPH expression levels were analyzed using One-way ANOVA.

Results

AMP Kinase-α Phosphorylation and ATP Levels: SF was not associated with changes in AMPK-α phosphorylation in either wild type or *gp91phox*$^{-/Y}$ mice (Figure 1 Online Supplement). Similarly, SF did not elicit significant changes in cortical ATP levels (Figure 1 Online Supplement)

Figure Legend

Figure E1. Effect of 15 days of sleep fragmentation on energy metabolism in the cortex in NADPH oxidase null and wild type mice. A. ATP levels in cortical tissues from *gp91phox*$^{-/Y}$ and C57BL6/J mice exposed to sleep fragmentation for 15 days or maintained under control sleep conditions (SC). There is no significant difference between the groups. (n=4/group)

B. Representative Western blots from showing the lack of AMPKα phosphorylation in the same cortical tissues from *gp91phox*$^{-/Y}$ and C57BL6/J mice exposed to sleep fragmentation for 15 days or maintained under control sleep conditions (SC). E10 epithelial cells exposed to 0.2% O_2 for 24 h were used as a positive control (+) for phosphorylated AMPKα.

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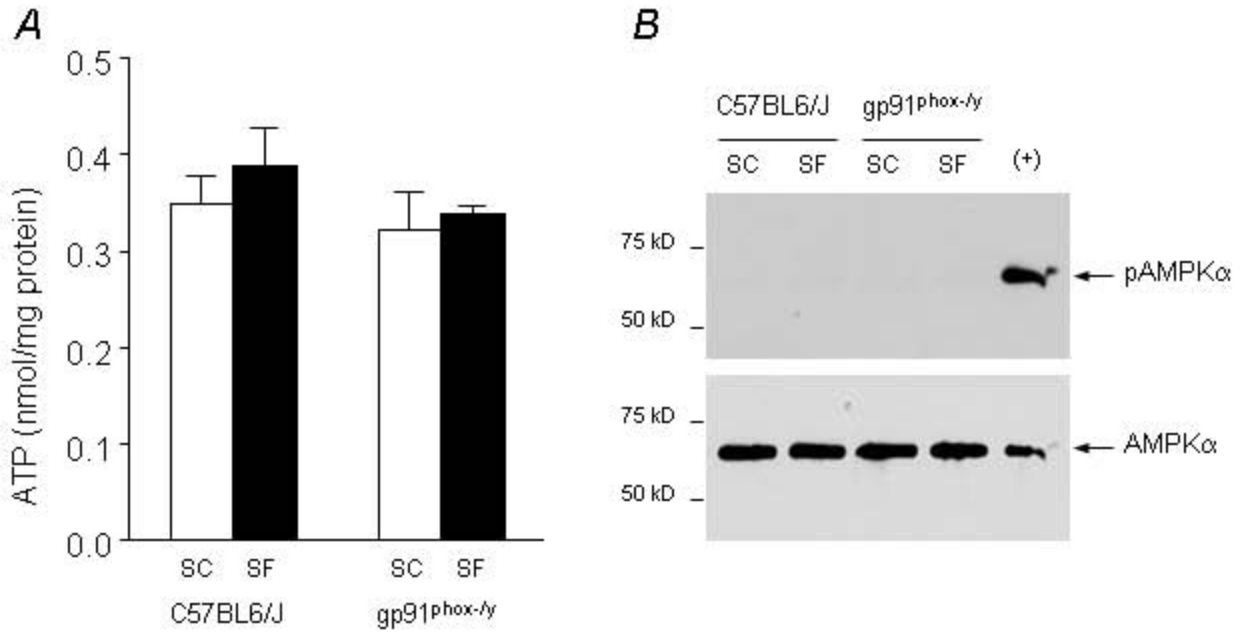
A
ATP (nmol/mg protein)
0.5
0.4
0.3
0.2
0.1
0.0
SC
SF
SC
SF
C57BL6/J
gp91phox-/y
B
C57BL6/J
gp91phox-/y
SC
SF
SC
SF
(+)
75 kD
50 kD
pAMPKα
75 kD
50 kD
AMPKα