

# Sleep Fragmentation Induces Cognitive Deficits Via Nicotinamide Adenine Dinucleotide Phosphate Oxidase-dependent Pathways in Mouse

Deepti Nair<sup>1\*</sup>, Shelley X. L. Zhang<sup>1\*</sup>, Vijay Ramesh<sup>1</sup>, Fahed Hakim<sup>1</sup>, Navita Kaushal<sup>1</sup>, Yang Wang<sup>1</sup>, and David Gozal<sup>1</sup>

<sup>1</sup>Department of Pediatrics, Pritzker School of Medicine, University of Chicago, Chicago, Illinois

**Rationale:** Sleep fragmentation (SF) is one of the major characteristics of sleep apnea, and has been implicated in its morbid consequences, which encompass excessive daytime sleepiness and neurocognitive impairments. We hypothesized that absence of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity is neuroprotective in SF-induced cognitive impairments.

**Objectives:** To examine whether increased NADPH oxidase activity may play a role in SF-induced central nervous system dysfunction.

**Methods:** The effect of chronic SF during the sleep-predominant period on sleep architecture, sleep latency, spatial memory, and oxidative stress parameters was assessed in mice lacking NADPH oxidase activity (*gp91phox*<sup>-/-</sup>) and wild-type littermates.

**Measurements and Main Results:** SF for 15 days was not associated with differences in sleep duration, sleep state distribution, or sleep latency in both *gp91phox*<sup>-/-</sup> and control mice. However, on a standard place training task, *gp91phox*<sup>-/-</sup> mice displayed normal learning and were protected from the spatial learning deficits observed in wild-type littermates exposed to SF. Moreover, anxiety levels were increased in wild-type mice exposed to SF, whereas no changes emerged in *gp91phox*<sup>-/-</sup> mice. Additionally, wild-type mice, but not *gp91phox*<sup>-/-</sup> mice, had significantly elevated NADPH oxidase gene expression and activity, and in malondialdehyde and 8-oxo-2'-deoxyguanosine levels in cortical and hippocampal lysates after SF exposures.

**Conclusions:** This work substantiates an important role for NADPH oxidase in hippocampal memory impairments induced by SF, modeling sleep apnea. Targeting NADPH oxidase, therefore, is expected to minimize hippocampal impairments from both intermittent hypoxia and SF associated with the disease.

**Keywords:** NADPH oxidase; sleep fragmentation; neurocognitive impairments

The manifestations of obstructive sleep apnea (OSA) reflect the interactions of intermittent hypoxia (IH), intermittent hypercapnia, increased intrathoracic pressure swings, and sleep fragmentation (SF) as elicited by the episodic changes in upper airway resistance during sleep. SF is a common phenomenon among several clinical

## AT A GLANCE COMMENTARY

### Scientific Knowledge on the Subject

Sleep apnea induces behavioral impairments in rodents partially because of increase in NADP reduced (NADPH) oxidase activity leading to oxidative stress. However, the contribution of sleep fragmentation to cognitive dysfunction and oxidative stress remains unknown.

### What This Study Adds to the Field

NADPH oxidase activity underlies components of sleep fragmentation-induced oxidative stress and cognitive impairments. These findings indicate that targeted therapy focused on NADPH oxidase activity may be useful in the treatment of patients with sleep disorders.

disorders, and can lead to impaired cognitive function via mechanisms that remain poorly understood (1). Indeed, uninterrupted sleep for a minimum length of time is required for optimal daytime vigilance and neurocognitive function (1–3). Preliminary studies in rodents using short-term SF paradigms have also confirmed the adverse effects of SF on learning and seem to be independent of adenosine-mediated synaptic inhibition (4–7).

In clinical populations with severe SF (e.g., in OSA) total sleep time typically diminishes only slightly (8). The effects of experimentally induced SF on sleep patterns have not been critically characterized in rodents. It is likely that OSA-induced sleep perturbations are accompanied by obvious cognitive deficits because of increased levels of systemic markers of oxidative stress and inflammation, the latter leading to gray matter loss in neural sites contributing to cognitive function. Thus, neural deficits could be mediated by IH, SF, or the interactions between these two disturbances (9–14). Several theories have suggested that sleep deprivation elicits oxidative stress cellular damage (i.e., the overall result of an imbalance between the reactive oxygen species [ROS] generated and clearance by the endogenous antioxidant defense system) (15) in discrete areas of the brain (16, 17), and that sleep decreases oxidative stress (18) by removing oxidants produced during waking time (19). Overall, it is generally accepted that sleep induces repair, restoration, and detoxification (20, 21). Conversely, sleep deprivation has been reported to cause oxidative stress resulting in the formation of ROS, and eventually could lead to neuronal and cellular damage (16). However, it remains unclear whether similar to sleep deprivation SF paradigms induce increased oxidative stress.

The primary catalytic function nicotinamide adenine dinucleotide phosphate (NADPH) oxidase was primarily studied in the context of its role in phagocyte oxidative burst (22). More recently, this enzyme has emerged as a major source of ROS generation in most mammalian cells, including neurons and synapses (23), either as

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Correspondence and requests for reprints should be addressed to David Gozal, M.D., Department of Pediatrics, Pritzker School of Medicine, Comer Children's Hospital, The University of Chicago, 5721 South Maryland Avenue, MC 8000, Suite K-160, Chicago, IL 60637. E-mail: dgozal@uchicago.edu

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a by-product of normal catalytic activity or as the result of aberrant function in disease (24–28). The authors have recently shown that the oxidative stress responses and neurobehavioral impairments induced by IH during sleep were partially mediated by excessive NADPH oxidase activity (14). However, it remains unknown whether NADPH oxidase activity is activated during SF, and whether such oxidative stress underlies putative SF-induced neurobehavioral dysfunctions. To explore this issue, NADPH oxidase mutant mice (*gp91phox*<sup>-Y</sup>) and wild-type littermates were exposed to SF and changes in sleep patterns, NADPH oxidase activity and expression, markers of oxidative stress, and hippocampus-dependent learning and memory tasks were all assessed. In addition, other behavioral paradigms for anxiety and depression were also tested, because such problems are frequently encountered in patients with sleep apnea (14, 29–34).

## METHODS

Figure 1 summarizes the experimental design for sleep, behavior, and biochemical studies. After each behavioral paradigm the mice were immediately returned to their respective exposures.

### SF Exposures

Male transgenic mice and control littermates were maintained in custom-made cages operated under a 12-hour light–dark cycle (7:00 AM to 7:00 PM) for 14 days before behavioral testing, and then for the duration of behavioral testing. The custom fabricated device used to induce SF in mice has been previously described (catalog #Model 80390; Lafayette Instruments, Lafayette, IN) (35).

### Surgical Procedure and Implantation of Telemetric Transmitter and Electrodes

All surgical procedures were performed under sterile conditions and general anesthesia. A telemetric transmitter weighing 3.5 g (F20-EET; DSI, St. Paul, MN), which allows simultaneous monitoring of two biopotential channels (temperature and locomotor activity), was chronically implanted to record the EEG from the frontal area and EMG from superior nuchal muscle (35). Sleep recordings, SF, and sleep scoring were done as previously described (35, 36).

### Behavioral Testing

In the Morris water maze the maze protocol is similar to that described by Morris and coworkers (37) with modifications for mice. Briefly, a standard place-training reference memory task was conducted on

mice in the water maze after exposure to 14 days of SF or sleep control (SC). Place learning was assessed over 6 consecutive days with three trials per day with an intertrial interval of 10 minutes (i.e., one block) using a spaced training regimen that has been demonstrated to produce optimal learning in mice (38). The performance in the water maze was assessed using mean escape latencies and swim distance. Retention tests were performed 14 days after acquisition of the task as previously described (14). For the elevated plus maze mice were placed in the center of the maze facing a closed arm, and allowed to explore for 10 minutes in isolation. The percent time spent in open and closed arms, number of entries to closed arms, and time spent in the center were analyzed. In the forced swimming test mice were individually forced to swim for 6 minutes on 2 consecutive days in an open cylindrical container (diameter 14 cm; temperature 25 ± 1°C). Mice were marked as immobile if they performed minimal amount of work required to float at least 1 second as previously described (14, 39, 40).

### Measurement of ATP Levels and Assessment of Adenosine Monophosphate Kinase $\alpha$ Activation

For more in-depth information see the online supplement.

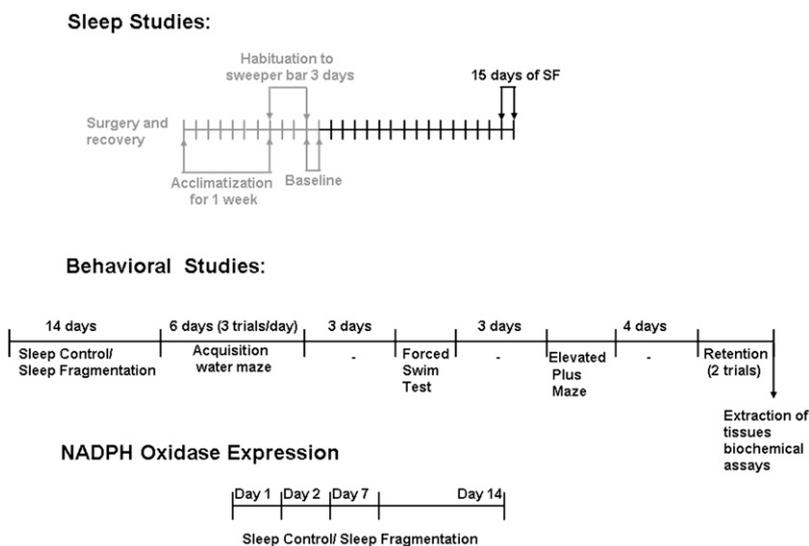
**NADPH oxidase expression.** The mRNA expression of p47phox and p67phox was determined by quantitative reverse transcriptase polymerase chain reaction using ABI PRISM 7500 System (Applied Biosystems, Foster City, CA) and commercially available specific primers (Applied Biosystems assays number Mm00447921\_m1 and Mm00726636\_m1, respectively).

**NADPH oxidase activity.** As previously described (41), cortical tissue homogenate incubated in assay buffer (200  $\mu$ l) containing acetylated cytochrome-c (100  $\mu$ M) in a 96-well plate at 30°C. NADPH (200  $\mu$ M) was then added in the absence or presence of superoxide dismutase (3 U/ $\mu$ l) and the reduction of cytochrome-c was monitored at 550 nm for 10 minutes. NOX activity was calculated as the superoxide dismutase-inhibitable reduction of cytochrome-c. All chemicals were from Sigma (St. Louis, MO).

**Lipid peroxidation and 8-OHDG assay.** For the assays, the frontal cortex and the hippocampus was dissected from mice and samples were processed for the lipid peroxidation malondialdehyde (MDA)-586 kits (OxisResearch, Portland, OR) and 8-oxo-2'-deoxyguanosine (8-OHDG) assay (Cell Biolabs, San Diego, CA). The assay was performed as described (14).

### Data Analysis

For sleep and behavioral tests, two-way repeated measures analysis of variance (ANOVA) followed by *post hoc* tests was used. For biochemical assays of MDA and 8-OHDG and NADPH expression levels were analyzed using one-way ANOVA. Statistics were performed using SPSS version 17.0 (IBM, Chicago, IL).



**Figure 1.** Schematic diagram illustrating sleep, behavior, and biochemical experiments with exposures to either sleep fragmentation (SF) or sleep control in both wild-type and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase null mice.

## RESULTS

### Sleep Measures

Overall analysis of the polysomnographic data for a period of 24 hours revealed significant changes between baseline (BL) and SF for wake ( $F = 22.57$ ;  $P < 0.001$ ), slow wave sleep (SWS) ( $F = 22.57$ ;  $P < 0.001$ ), and REM sleep ( $F = 22.57$ ;  $P < 0.001$ ), and delta power during SWS ( $F = 22.57$ ;  $P < 0.001$ ) indicating there was a significant effect on light period versus dark period. SF increased wake episodes ( $F = 52.563$ ;  $P < 0.001$ ) and reduced SWS latency ( $F = 7.643$ ;  $P < 0.002$ ), indicating that SF continued to elicit the desired frequency of awakenings and increased sleep propensity.

During the light period, C57BL6/J mice were awake for  $38.52 \pm 4.11\%$  and  $gp91^{-/-}$  mice were awake for  $33.19 \pm 1.92\%$  of the time (Figure 2A). During the dark period, C57BL6/J mice were awake for  $64.59 \pm 3.67\%$  and  $gp91^{-/-}$  mice were awake for  $64.69 \pm 1.25\%$  of the time. Thus, no differences emerged between C57BL6/J and  $gp91^{-/-}$  mice (Figure 2). Similar findings occurred for SWS (Figure 2B) and REM sleep (Figure 2C). However, there were significant, albeit minor, differences in REM sleep between C57BL6/J at BL and C57BL6/J after SF ( $q = 4.22$ ;  $P < 0.04$ ) during the light period, and minor differences in REM sleep during the dark period (C57BL6/J BL vs. C57BL6/J SF [ $q = 4.10$ ;  $P < 0.04$ ]; C57BL6/J BL vs.  $gp91^{-/-}$  SF [ $q = 6.06$ ;  $P < 0.003$ ]; and  $gp91^{-/-}$  BL vs.  $gp91^{-/-}$  SF [ $q = 5.30$ ;  $P < 0.009$ ]).

### Delta Power during SWS

No changes in delta power between C57BL6/J mice and  $gp91^{-/-}$  mice emerged in SF (Figure 2D).

### Wake Episodes

During the light period there were significant changes in wake episodes between C57BL6/J BL and C57BL6/J SF ( $q = 17.70$ ;

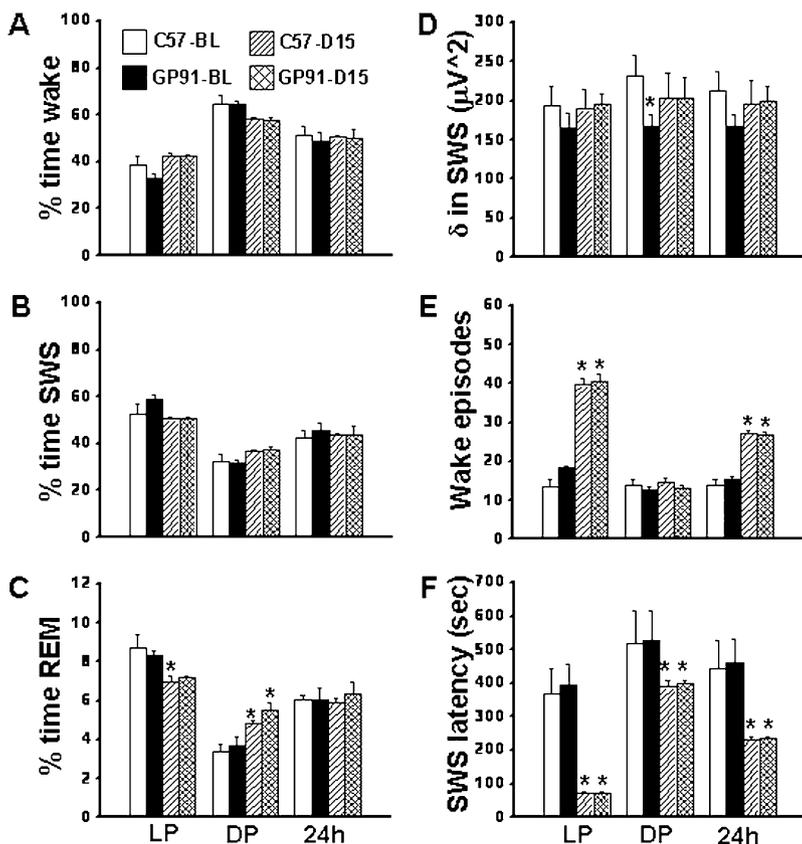
$P < 0.001$ ) and between  $gp91^{-/-}$  BL and  $gp91^{-/-}$  SF ( $q = 15.09$ ;  $P < 0.001$ ), as targeted by the SF paradigm (Figure 2E). There were no changes in wake episodes during the dark period. As a result, there were significant increases in wake episodes for the 24-hour period in SF for C57BL6/J and  $gp91^{-/-}$  mice (Figure 2E).

### SWS Latency

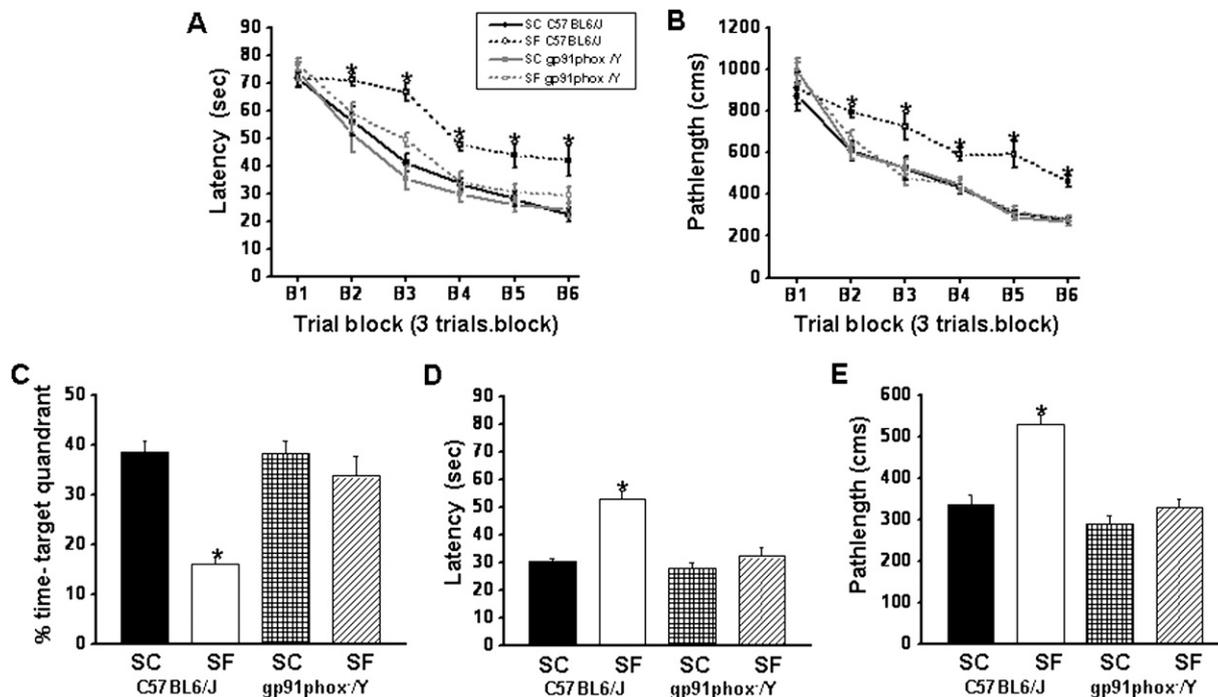
Significant decreases in SWS latency emerged in all SF-exposed mice during the light period (C57BL6/J BL vs. C57BL6/J SF,  $q = 7.307$ ,  $P < 0.001$ ;  $gp91^{-/-}$  BL vs.  $gp91^{-/-}$  SF,  $q = 7.97$ ,  $P < 0.001$ ), but no differences between mouse strains were apparent (Figure 2F). Reductions in SWS latency were also identified during the dark period and the total 24-hour period for both mouse strains after SF (Figure 2F).

### Spatial Learning Performance

On the standard place discrimination task, wild-type mice exposed to 14 days of SF (SF-C57BL6/J) exhibited longer latencies and pathlengths to locate the hidden platform when compared with either control animals (SC-C57BL6/J, SC- $gp91^{phox^{-/-}}$ ) or with  $gp91^{phox^{-/-}}$  mice exposed to 14 days SF (SF- $gp91^{phox^{-/-}}$ ;  $n = 12$  per experimental condition) (Figures 3A and 3B). Overall latency analysis for the entire trial blocks revealed significant changes between the different treatment groups ( $F = 26.128$ ;  $P < 0.001$ ) and pathlength ( $F = 28.605$ ;  $P < 0.001$ ) indicating that SF adversely affected task performance. Significant differences in latencies were observed during blocks 2 ( $F = 2.90$ ;  $P < 0.049$ ); 3 ( $F = 18.136$ ;  $P < 0.001$ ); 4 ( $F = 6.811$ ;  $P < 0.001$ ); 5 ( $F = 7.279$ ;  $P < 0.001$ ); and 6 ( $F = 5.421$ ;  $P < 0.004$ ). There were no significant differences in Block 1. Repeated measures ANOVA revealed significant differences in path lengths during blocks 2 ( $F = 6.875$ ;  $P < 0.001$ ); 3 ( $F = 4.524$ ;  $P < 0.009$ ); 4 ( $F = 7.832$ ;  $P < 0.001$ ); 5 ( $F = 17.465$ ;  $P < 0.001$ ); and 6 ( $F = 20.417$ ;  $P < 0.001$ ), with no significant differences in Blocks 1 and 2.



**Figure 2.** Sleep patterns in mice exposed to sleep fragmentation (SF). There were no changes in total wake (A), slow wave sleep (SWS) (B), REM sleep (C), and delta power (D). An increase in the number of wake episodes (E) and decreases in SWS latency (F) after 15 days of SF are apparent.  $n = 6$  per experimental group. \*  $P < 0.05$ ; see text for more details.



**Figure 3.** *gp91phox*<sup>-/-</sup> mice exposed to sleep fragmentation (SF) do not exhibit any deficits in acquisition and retention. (A and B) Mean latencies (s) and pathlengths (cm) to locate the target platform during place training in C57BL6/J and *gp91phox*<sup>-/-</sup> either exposed to SF or sleep control (SC) ( $n = 12$  per group). (C) Mean percentage time in the target quadrant during probe trial after completion of water maze testing in either C57BL6/J and *gp91phox*<sup>-/-</sup> exposed to SF or SC ( $n = 12$  per group). (D) Mean latencies (s) and (E) pathlengths (cm) to locate the target platform during retention in C57BL6/J and *gp91phox*<sup>-/-</sup> either exposed to SF or SC during assessment of retention of the Morris water maze task.  $n = 12$  per group. \*  $P < 0.05$ .

There were no significant differences in swim speed in these mice (data not shown). In the probe-trial test, one-way ANOVA revealed a significant effect of treatment (SF vs. SC:  $F = 23.219$ ;  $P < 0.001$ ). The magnitude of impairment was present only in SF-C57BL6/J mice (Figure 3C). In the reference memory tests, SF-C57BL6/J mice exhibited significant deficits in memory retention in both latency ( $F = 23.454$ ;  $P < 0.001$ ) and pathlength ( $F = 20.189$ ;  $P < 0.001$ ). However, the SF-*gp91phox*<sup>-/-</sup> mice performed similar to control animals (Figure 3D and 3E).

Repeated measures multivariate ANOVA with latency, groups, and conditions ( $F_{[5,39]} = 44.13$ ;  $P < 0.0001$ ) revealed that SC C57BL6/J mice required significantly less time than their littermates exposed to SF to find the hidden platform in a Morris water maze (Figure 3A), whereas the SF-*gp91phox*<sup>-/-</sup> mice were not affected. Repeated measures multivariate ANOVA with pathlength, groups, and conditions ( $F_{[5,39]} = 15.304$ ;  $P < 0.0001$ ) indicated that as the training progressed the SC *gp91phox*<sup>-/-</sup> and SC C57BL6/J mice could reach the hidden platform and covered the shortest distance compared with the distance covered by C57BL6/J mice exposed to SF (Figure 3B). In addition, repeated measures multivariate ANOVA with swim speed, groups, and conditions on the swim speed showed no significant differences between the groups and treatments (data not shown).

#### Elevated Plus Maze

SF-C57BL6/J mice showed significant differences in the percentage of time spent in the open arm ( $F = 74.625$ ;  $P < 0.001$ ) and in the number of entries into the closed arm ( $F = 13.018$ ;  $P < 0.001$ ) (Figures 4A and 4B). The results of the elevated plus maze showed that SF-C57BL6/J spent significantly less time in the open arms (Figure 4A) (group effect,  $F = 29.903$ ;  $P < 0.0001$ ) and significantly more time in the center area (Figure 4C) (group effect,  $F = 9.277$ ;  $P < 0.0001$ ). The number of entries into the

closed arms was significantly increased (Figure 4B) (condition effect,  $F = 9.899$ ;  $P < 0.0001$ ). Although the percentage of time spent in the open arm is commonly used as a measure of anxiety, the time spent on the center platform of the maze and the closed arm entries all reflect anxiety-like behaviors in mice.

#### Forced Swim Test

SF-C57BL6/J mice had significantly higher immobility durations during the last 4 minutes of the forced swim test ( $F = 5.614$ ;  $P < 0.004$ ) compared with all other treatment groups, including SF-*gp91phox*<sup>-/-</sup> (Figure 4D), suggesting the presence of depressive-like behaviors.

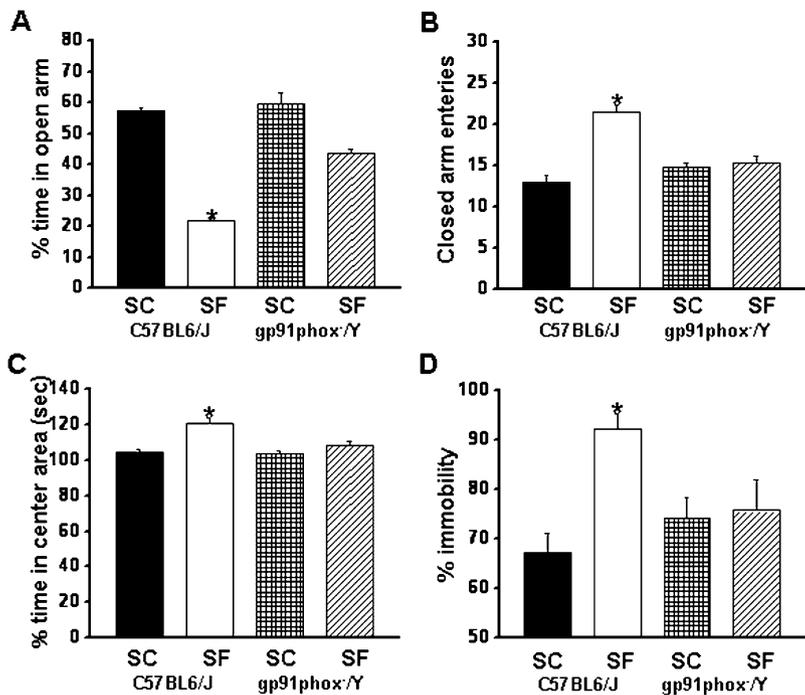
#### NADPH Oxidase Expression and Activity

SF induced increased expression of the P47phox and P67phox subunits of the NADPH oxidase gene (Figures 5C and 5D). Furthermore, SF-exposed wild-type mice showed NADPH oxidase activity increases, which were absent in *gp91phox*<sup>-/-</sup> mice (Figure 5B).

#### Lipid Peroxidation and 8-OHDG Levels

After the behavioral experiments, cortical tissues and hippocampus were harvested and processed for assessment of lipid peroxidation as indicated by MDA levels. Figures 6A and 6B show MDA concentrations in homogenates of cerebral cortex from all treatment groups. A significant increase in MDA levels was observed in SF-C57BL6/J mice in the cortex ( $F = 14.051$ ;  $P < 0.001$ ) and in the hippocampus ( $F = 34.22$ ;  $P < 0.001$ ) compared with all three other experimental groups.

The levels of 8-OHDG in homogenates of cerebral cortex and the hippocampus were significantly higher in SF-C57BL6/J mice ( $F = 68.588$ ,  $P < 0.001$ ; and  $F = 13.392$ ,  $P < 0.001$ ), respectively,



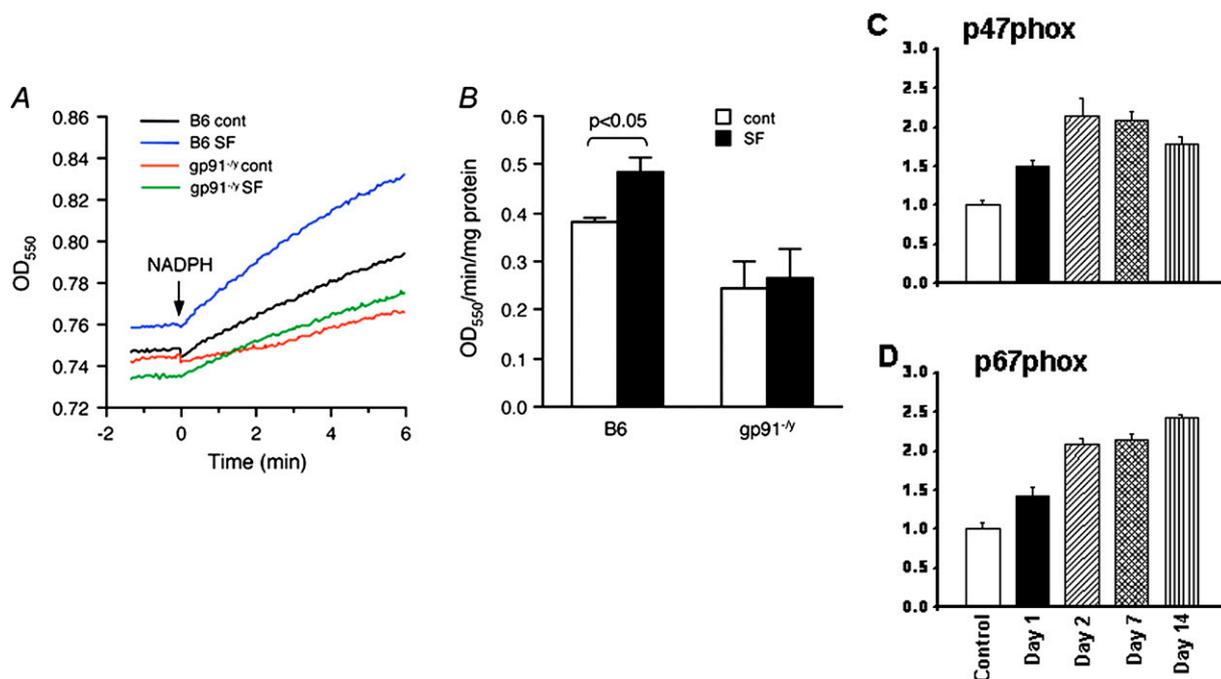
**Figure 4.** Exposure to sleep fragmentation (SF) induces anxiety and depression in mice, which is abrogated in NADP reduced oxidase null mice. (A) C57BL6/J mice exposed to SF spent significantly less time in the open arm of the elevated plus maze compared with sleep control (SC) C57BL6/J, or with *gp91phox*<sup>-/-</sup> mice exposed to SF or maintained under SC. (B) An increased number of closed-arm entries emerged in wild-type mice exposed to SF. (C) Time spent in the Center Area was increased in wild-type mice exposed to SF. n = 12 per group; \* P < 0.05. (D) *gp91phox*<sup>-/-</sup> exposed to SF show less immobility compared with C57BL6/J mice exposed to SF. n = 12 per experimental group. \* P < 0.05; see text for more details.

compared with all other groups (Figures 6C and 6D). However, there were no significant differences in the levels of 8-OHDG in cortex of SF-*gp91phox*<sup>-/-</sup> compared with control animals SF-*gp91phox*<sup>-/-</sup> and SF-C57BL6/J.

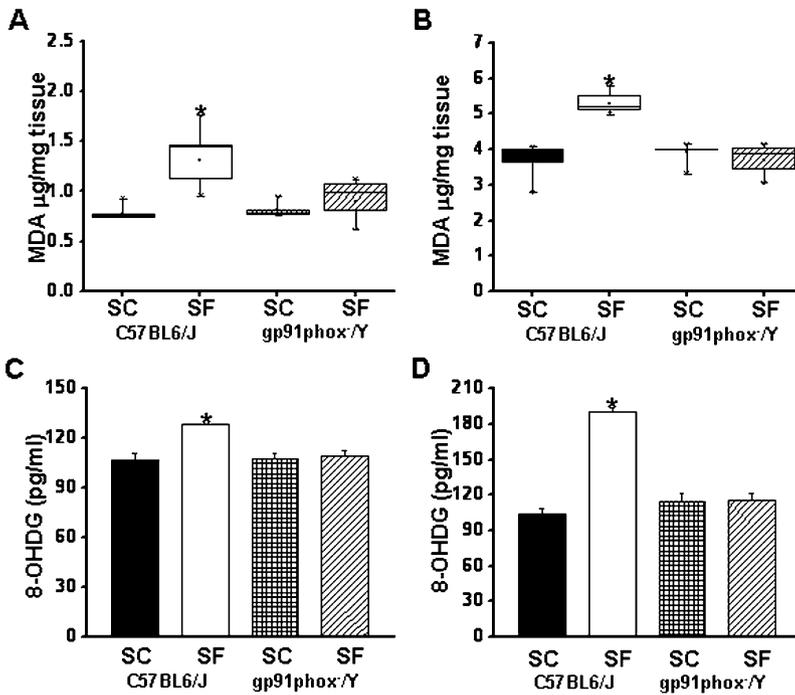
**DISCUSSION**

The present study demonstrates that 12 hours of SF during the daylight period for 14 days, which aims to mimic sleep apnea,

interferes with spatial learning and retention memory tests in a well-defined hippocampus-dependent learning and memory task (14, 42). The SF-induced performance deficits in the water maze could be mediated by a disturbance in hippocampal-dependent memory consolidation. In addition, chronic SF is not accompanied by prominent differences in sleep state distribution and duration, and yet the recurrent arousals induced during daylight hours are manifest as marked decreases in sleep latency (i.e., increased sleep propensity). Current



**Figure 5.** Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and expression. NADPH oxidase activity in the cerebral cortex. (A) Kinetic NADPH oxidase activity measured as NADPH-dependent cytochrome-c reduction. Shown are representative tracings from the four experimental groups. (B) Summary of NADPH oxidase activities. Sleep fragmentation (SF) resulted in a significant increase in NADPH oxidase activities in wild-type mice. Such SF-induced increases in NADPH oxidase activity were abolished in *gp91phox*<sup>-/-</sup> mice. n = 4 for each group. Data are mean ± SE. (C and D) Expression of p47phox and p67phox subunits of NADPH oxidase was significantly increased during the course of SF in C57BL6/J mice.



**Figure 6.** Lipid peroxidation and oxidative DNA damage after sleep fragmentation (SF). Lipid peroxidation is reduced in the frontal cortex (A) and hippocampus (B) of *gp91phox*<sup>-/-</sup> mice exposed to SF. Malondialdehyde tissue levels of *gp91phox*<sup>-/-</sup> and C57BL6/J mice exposed to SF or control sleep conditions (SC) for 14 days. n = 6 per experimental group; \* *P* < 0.05. Oxidative DNA damage is absent in the cortex (C) and hippocampus (D) of *gp91phox*<sup>-/-</sup> mice exposed to SF. 8-oxo-2'-deoxyguanosine levels in cortex and hippocampus of *gp91phox*<sup>-/-</sup> and C57BL6/J mice exposed to SF or SC for 14 days. n = 6 per experimental group; \* *P* < 0.05. MDA = ; OHdG = .

findings also point out for the first time that chronic SF induces increased expression and activation of NADPH oxidase, but that the SF-induced oxidative stress that is abrogated in NADPH oxidase null mice does not seem to play a role in the emergence of increased sleep propensity, because both SF-*gp91phox*<sup>-/-</sup> and SF-C57BL6/J showed similar reductions in SWS latencies. However, excessive NADPH oxidase activity likely mediated the cognitive and behavioral deficits elicited by chronic SF.

During sleep, patients with sleep apnea are subjected to recurrent and chronic SF and IH, both of which induce substantial increases in NADPH oxidase expression and activity, and ROS production and consequent cellular dysfunction (43). In the context of sleep perturbations and learning, most studies have reported on the presence of cognitive impairments with sleep deprivation by exploring a large array of learning and memory tests including the radial arm maze (44, 45), Morris water maze (6, 46), contextual fear conditioning (47), and eight-box task (48). However, there is only a paucity of cognitive studies exploring the effects of SF, and those were restricted to short-term SF, the latter using methodologies that were associated with substantial alterations in sleep architecture (4–6). In addition, we postulate that because the SF-exposed mice exhibited overall preserved sleep architecture, the fact that SF-*gp91phox*<sup>-/-</sup> mice were protected against SF-induced cognitive deficits is highly unlikely to be explained by any underlying disruption of sleep architecture, or by reduced brain energy source bioavailability. This is particularly pertinent when considering the preserved ATP levels and absence of any phosphorylation of AMPK- $\alpha$  (see online supplement), whereas both of these reporters of cellular bioenergetics are markedly altered as a consequence of sleep deprivation (49). Consequently, it is assumed that NADPH-mediated ROS were the major contributor to the disrupted neurobehavioral and cognitive manifestations of SF. ROS can be generated from various subcellular compartments, including mitochondria, the cellular membrane, lysosomes, peroxisomes, and the endoplasmic reticulum (27, 50, 51). Certain levels of ROS are extremely important for a variety of normal biologic functions including growth factor regulation, calcium

signaling, and phagocytosis/inflammation (52–54). For example, NADPH oxidase (27, 55, 56), xanthine oxidase (57), phospholipase A<sub>2</sub> (57), lipoxygenases and cyclooxygenase (50), and cytochrome P-450 (58) have all been identified as sources of ROS in various subcellular compartments under both physiologic and pathologic conditions. Indeed, physiologic concentrations of hydrogen peroxide seem to be essential to the integrity of hippocampal long-term potentiation (59–61), whereas increased concentrations of the hydrogen peroxide are markedly detrimental to hippocampal function (62). Similar inferences regarding other ROS sources including NADPH oxidase biologic functions have been reported (63–65). Thus, excessive ROS bioavailability, such as that generated by SF-induced increased expression and activation of the various subunits of NADPH oxidase that are present in cortical and hippocampal neurons (25, 26, 66, 67), results in deleterious effects on the performance in a spatial task acquisition and retention, and also underlies the changes in behavior, as measured in the forced swim test (depression) or the elevated plus maze (anxiety). Although it cannot be inferred from the current findings on the cellular source of NADPH oxidase contribution to SF-induced cognitive and behavioral deficits, the near complete abrogation of such deficits in the *gp91phox*<sup>-/-</sup> mice clearly and conclusively assigns a critical role for NADPH oxidase in this context. Of note, activation of NADPH oxidase and consequent ROS production and oxidative stress can also occur in sleep apnea, particularly in the context of IH, and may mediate components pulmonary vasoconstriction (68, 69), chemoreceptor hypersensitivity (70), and mobilization of progenitor cells (71).

Depressive and anxiety symptoms are frequent in OSA patients (34, 72, 73), and in the present study some of these frequent clinical complaints are reproduced in the model of chronic SF, suggesting that the recurrent arousals and increased oxidative stress play a role in the depressive and anxiety symptoms of the disease. Indeed, depression may account for the fatigue seen in OSA patients, even after adjusting for OSA severity (31). Some studies have reported higher responsiveness of hippocampus and hypothalamus and reduced susceptibility to oxidative stress in cortex and brainstem in response to sleep

deprivation. Conversely, others have reported on the presence of increased oxidative stress in the hippocampus and cortex in sleep-deprived rats (74–76). The divergent reports are perhaps caused by the inherently different models of sleep deprivation used and also by the different brain regions being assessed. Notwithstanding such considerations, ROS have been implicated in the pathophysiology of depression and anxiety (77). The elevated plus-maze is the most frequently used animal model for assessing anxiety-like behaviors (78, 79) because it enables researchers to observe the conflict between two innate rodent behaviors: the avoidance of open space exposure as countering the tendency to explore novel environments (79). The results further imply that SF modified anxiety-like behaviors in wild-type mice. In contrast, *gp91phox*<sup>-Y</sup> exposed to SF showed preserved performances in this test, suggesting that regions underlying these behavioral patterns are susceptible to SF, most likely via the oxidant stress mediated by activation of NADPH oxidase.

In summary, the current findings on chronic SF-induced oxidative stress (Figure 6) pave the way for a viable rationale supporting the use of antioxidant therapies aiming to reduce the excessive ROS generation, accumulation, and propagation, and thus palliate the neurocognitive and mood and behavioral dysfunction associated with sleep apnea. Indeed, transgenic mice null for NADPH oxidase activity displayed significantly reduced lipid peroxidation and DNA damage after SF exposures, and preserved cognition and behavior (Figures 3 and 6). The present study used a combination of sleep recordings, and behavioral, molecular, and biochemical studies, and demonstrated that oxidative stress induced by excessive NADPH oxidase-mediated superoxide release is a critically important mediator of the neuronal dysfunction induced by SF, but is not involved in the mechanisms underlying SF-induced excessive sleepiness.

**Author disclosures** are available with the text of this article at [www.atsjournals.org](http://www.atsjournals.org).

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