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REM sleep deprivation increases the expression of interleukin genes in mice hypothalamus

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HIGHLIGHTS

- We investigated the change of inflammatory gene expressions by REM SD in the hypothalamus.
- Among IL subfamily genes, REM SD increased most potently IL1β gene expression.
- IL subfamily genes, and in particular IL1β, might be involved in sleep regulation.

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ABSTRACT

Recently, evidence has suggested the possible involvement of inflammatory cytokines in sleep deprivation (SD). In this study, we assessed the patterns of inflammatory gene regulation in the hypothalamus of REM SD mice. C57BL/6 mice were randomly assigned to two groups, SD ($n = 15$) and control groups ($n = 15$). Mice in the SD group were sleep-deprived for 72 h using modified multiple platforms. Microarray analysis on inflammatory genes was performed in mice hypothalamus. In addition, interleukin 1 beta (IL1β) protein expression was analyzed by the immunochemistry method. Through microarray analysis, we found that expressions of IL subfamily genes, such as IL1β (2.55-fold), IL18 (1.92-fold), IL11 receptor alpha chain 1 (1.48-fold), IL5 (1.41-fold), and IL17E genes (1.31-fold), were up-regulated in the hypothalamus of SD mice compared to the control. The increase in the expression of these genes was also confirmed by RT-PCR. Among these genes, the expression of IL1β was particularly increased in the hypothalamus of SD mice. Interestingly, we found that the protein expression of endogenous IL1β was also elevated in the hypothalamus of SD mice compared to the control mice. These results implicate that IL subfamily genes, and in particular, IL1β, may play a role in sleep regulation in the hypothalamus of REM SD mice.

1. Introduction

Sleep is a required process of the brain for proper functioning, and it is also important in the optimal homeostasis of the emotional brain function. Sleep is important for maintaining synaptic balance, and wakefulness is responsible for synaptic potentiation in cortical circuits [5]. Sleep deprivation (SD) has been demonstrated by increased pro-inflammatory cytokines, appetite, and blood pressure as well as cortisol levels [2,16]. In addition, SD was known to cause negative effects on emotional behavior, attention, learning, and memory, and also may subsequently give rise to psychiatric disorders.

Cytokines, mediators of immune system responses, were implicated to interact with the sleep mechanisms of the brain. Cytokines were known to signal the central nervous system (CNS) in order to regulate normal sleep patterns, alter sleep during infectious disease and pathology, and induce the altered behavior and symptoms associated with the illness [13]. Several studies have reported the relationship between SD and cytokines. Pro-inflammatory cytokines, such as interleukin 1 beta (IL1β), IL6, and tumor necrosis factor-α (TNF-α), have been thoroughly evaluated to contribute to sleep regulation in brain regions, such as the hypothalamus and the hippocampus [28]. Diurnal variations in concentrations of pro-inflammatory cytokines, such as IL1β and TNF-α, were reported to be associated with the sleep regulation [9,28]. In another study, SD has been reported to increase the plasma levels of IL1β and IL1 receptor antagonist [3]. In addition,

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IL1, IL2, IL6, IL8, IL18, and TNF-α were known to promote non-rapid eye movement (NREM) sleep [10,18], whereas IL4, IL10, IL13, and transforming growth factor-β suppressed NREM sleep [6].

The hypothalamus is now recognized as the most important brain site for the sleep switch, and as a key regulator of sleep and wakefulness. Sleep rhythm is controlled by a circadian pacemaker located in the hypothalamus, which regulates mood, appetite, sexual drive, circadian rhythms, and endocrine functions [1]. It has been shown that SD resulted in hypothalamic-pituitary-adrenal axis activation, and augmented the immunoreactivity for corticotrophin-releasing hormone in the paraventricular nucleus (PVN) of the hypothalamus [4]. Mackiewicz et al. [14] assessed changes in gene expression in the cerebral cortex and hypothalamus of the mouse following SD, and reported the alteration of cell growth-, cytoskeleton organization-, and biogenesis-related genes in the hypothalamus. Despite the potential roles of cytokines in SD and hypothalamus in sleep regulation, the study about cytokine regulation in the hypothalamus by SD has not been deeply investigated so far. Thus, we investigated the change of inflammatory gene expressions by SD in the hypothalamus using REM SD mice to characterize the transcript profile of inflammatory genes resulting from SD.

2. Materials and methods

2.1. Animals and modified multiple platform method

Ten week-old male C57BL/6 mice (24–29 g; Central Lab. Animal Inc., Korea) were housed under a 12-h light/dark cycle at a standard temperature (22 ± 3 °C) with food and water freely available. All experimental procedures were carried out according to the animal care guidelines of the National Institute for Health (NIH) Guide and the Korean Academy of Medical Sciences.

SD was conducted using the multiple platform method, modifying the previously described multiple platform method [21] that is a widely used method for REM SD [30]. Eleven week-old male C57BL/6 mice were used for this study. Mice were randomly assigned to two groups, the SD (n = 15) and the control groups (n = 15). Mice (5 mice per tank cage) in the SD group were placed in water tanks (56 cm × 40 cm × 19 cm), containing 15 circular platforms (3 cm diameter) each, surrounded by water up to 1 cm beneath the surface, for 72 h. When they reached the paradoxical phase of sleep, muscle atonia caused them to fall into the water and wake up. In each tank, mice were coming from the same cage where they were previously housed, and were capable of moving inside and jumping from one platform to the other. Mice (5 mice per tank cage) in the control group were submitted to the same procedure, except that the platforms were 8.5 cm in diameter. Platforms, which were surrounded by water, were large enough for the mice to sleep on, but still not large enough for the mice to walk around on. Throughout the study, mice were housed under a 12-h light/dark cycle at a standard temperature (22 ± 3 °C). Food and water were made available ad libitum through a grid placed on top of the water tank.

2.2. RNA extraction

Mice (n = 6 per group) were anesthetized using CO_2 and were decapitated. We collected brains from both groups, and isolated the total hypothalamus from each mouse brain. Total RNA from the isolated hypothalamus was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions, and was purified using an QIAGEN RNeasy kit (QIAGEN Sciences, Germantown, MD). RNA integrity was evaluated using a ND-1000 UV–vis spectrophotometer (Nanodrop Technologies Inc., Montchanin, DE) to measure the absorbance ratio (A260/A280). RNA samples were additionally analyzed with agarose gel electrophoresis and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) to evaluate the integrity of the RNA.

2.3. Microarray analysis

For gene expression analysis, CombiMatrix 4 × 2K mouse Inflammation chip (Digital Genomics, Seoul, South Korea) was used. Fluorescently labeled probes for oligo microarray analysis were prepared by Amino allyl MessageAmp™ mRNA kit (Ambion Inc., Austin, TX). The labeled probes were hybridized at 60 °C for 16 h. Slides were washed twice in 6× SSC/0.005% Triton X-100 at 60 °C for 20 min, once in 0.1× SSC/0.005% Triton X-100 at RT for 10 min, and four times in D.W. for 1 min and then spin dried. DNA chips were scanned using Scan Array Lite (Perkin–Elmer Life Sciences, Billerica, MA). Scanned images were analyzed with the GenePix 3.0 software (Axon Instruments, Union, CA) in order to obtain the gene expression ratios. After normalizing the data, genes with a higher or lower expression ratio were selected. The expression of 1.3 was set as the cut-off value. The logged gene expression ratios were normalized by LOWESS regression.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

To synthesize the cDNA, extracted RNA samples and random hexamers (Promega, Madison, WI) were added together, and the mixture was heated for 10 min at 70 °C. The reaction mixture was cooled to the RT and then transferred on ice. AMV reverse transcriptase, 10 mM each with dNTP, RNasin, and 5× RT reaction buffer were added; the final volume was brought up to 30 μl with diethyl pyrocarbonated treated water. Reverse transcription was performed by incubating the mixture at 37 °C for 60 min and then at 95 °C for 5 min. Subsequent PCR amplification was performed in a reaction volume of 20 μl containing the appropriate cDNA, each set of primers at a concentration of 10 pM, 10× reaction buffer, 2.5 mM dNTP, and 2 U of Taq DNA polymerase. The RT-PCR products were electrophoresed and visualized by staining with ethidium bromide. The results of the RT-PCR were quantified by using the ImageJ image analysis software (NIH, Bethesda, MD, USA).

2.5. IL1β immunohistochemistry

Mice (n = 9 per group) were killed and transcardially perfused with 0.05 M phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were removed and post-fixed overnight, and then cryoprotected overnight in 30% sucrose solution for 48 h. The brains were coronally sectioned at 40 μm using a freezing microtome (Leica, Nussloch, Germany). Floating sections, involving the hypothalamus area, were incubated for 15 min in 3% hydrogen peroxide to eliminate endogenous peroxidase activity. Next, the floating sections were blocked in 0.05 M PBS containing 10% normal goat serum and 1% bovine serum albumin (BSA) for 1 h, and were incubated with rabbit anti-IL1β antibody (Santa Cruz Biotechnology, Santa Cruz, CA) (1:500 dilution in PBS containing 1% BSA) overnight. Then, they were incubated with biotinylated-conjugated goat anti-rabbit IgG for 1 h, and then incubated with an avidin–horseradish peroxidase complex (Elite ABC kit, 1:200, Vector Laboratories, Burlingame, CA) for another 1 h. Finally, the sections were stained with diaminobenzidine, mounted on a gelatinized glass slide and dehydrated. Immunohistochemical images were visualized using a light microscope (Olympus, Tokyo, Japan). Quantification was performed at ×200 using a computer image analysis system, Metaview (Media Cybernetics, Silver Spring, MD). The immunoreactivity level of IL1β was measured quantitatively as a percentage thresholded area on the hypothalamus.

Table 1
Interleukin subfamily genes up-regulated by REM sleep deprivation (SD) in the hypothalamus.

Accession no.	Gene – symbol	Title	Fold change (SD/control)
NM_008361	IL1β	Interleukin 1 beta	2.55
NM_008360	IL18	Interleukin 18	1.92
NM_010549	IL11RA1	Interleukin 11 receptor, alpha chain 1	1.48
NM_153077	IL1F10	Interleukin 1 family, member 10	1.45
NM_172786	IL20RA	Interleukin 20 receptor, alpha	1.42
NM_010558	IL5	Interleukin 5	1.41
NM_080729	IL17E	Interleukin 17e	1.31
NM_009909	IL8RB	Interleukin 8 receptor, beta	1.30

2.6. Statistical analysis

All values are presented as mean ± standard error of the mean (S.E.M.). Statistical analysis was performed by a Student's *t*-test using the SPSS software (release 18.0; SPSS Inc., Chicago, IL, USA). Differences between the groups with values of $p < 0.05$ were considered as significant.

3. Results

3.1. RNA isolation and analysis of microarray expression data

The RNA from the mice brain hypothalamus tissues was isolated and validated for its quality. The result presented good qualities of isolated RNAs prepared for chip hybridization (Supplementary data 1). The green spots represent the genes of the control group, labeled by Cy3-captured reagent; and they are overexpressed than those of the SD group. In contrast, the red spots represent the genes of the SD group, labeled by Cy5-captured regent; and they are overexpressed than those of the control group. The yellow spots represent the genes that showed no difference in expression level between the control and the SD groups (Supplementary data 2).

3.2. Expression of IL genes

On the CombiMatrix 4 × 2K Mouse Inflammation chip, 128 inflammation genes from the hypothalamus of SD mice were increased more than 1.3-fold compared to those of the control mice (Supplementary data 2). Among the increased genes, we found that SD particularly up-regulated the expression of genes belonging to the IL subfamily. Table 1 shows the expression change of IL subfamily genes in the hypothalamus of SD mice. Interestingly, SD up-regulated the expression of IL1β gene more than two-fold (2.55-fold). We also observed the increase of IL18 (1.92-fold), IL5 (1.41-fold), and IL17E gene expressions (1.31-fold) in the hypothalamus of SD mice. Moreover, SD elevated the expression of IL receptor genes. In SD mice compared to the control mice, the expression of IL11 receptor alpha chain 1 (IL11RA1), IL20RA, and IL8 receptor beta (IL18RB) genes increased 1.48-fold, 1.42-fold, and 1.30-fold, respectively (Table 1). However, the level of any gene did not decrease more than 1.3-fold in the hypothalamus of SD mice.

3.3. Confirmation of IL genes

To confirm the microarray findings, we assessed the mRNA expression of IL genes, such as IL1β, IL18, IL5 and IL17E, as well as IL receptor gene, such as IL11RA1, by RT-PCR using primers for each gene: Cyclophilin (sense, 5′-ACCCCACCGTGTTCTTCGAC-3′; antisense, 5′-CATTTGCCATGGACAAGATG-3′), IL1β (sense, 5′-GAGAGTGTGGATCCCAAGCAATAC-3′; antisense, 5′-GTCTCCTA-GAGATTGAGCTGTCTGC-3′), IL18 (sense, 5′-GTGAAGTAAGAGGACT-GGCTGTGAC-3′; antisense, 5′-CCCTCCCCACCTAACTTTGATGTA-3′), IL11RA1 (sense, 5′-ACCCGCTACCTTACTTCCTACAG-3′; antisense, 5′-CTCCTGAGCTACTACTGCCTCTAGC-3′), IL5 (sense, 5′-CTTGGTG-TGATGAGTACAGAGTGG-3′; antisense, 5′-GGGAGGGAGTATAACT-CAGTTGGT-3′) and IL17E (sense, 5′-GAGCTATGAGTTGGAC-AGGGACT-3′; antisense, 5′-GTCCGTTCCTGAGTAGAAATGC-3′). As shown in Fig. 1, the mRNA expression of these genes showed a similar pattern to those of the microarray data presented in Table 1. The mRNA expression of IL1β increased most prominently (2.50 ± 0.08-fold) in the hypothalamus of SD mice compared to the control mice. The expression of IL18 (1.78 ± 0.26-fold), IL5 (1.27 ± 0.02-fold), IL17E (1.71 ± 0.12-fold), and IL11RA1 (1.69 ± 0.19-fold) genes was also elevated in SD mice. The efficiency of the reaction was adjusted by Cyclophilin amplification (Fig. 1).

Among IL subfamily genes, SD increased most potently the IL1β gene expression (Table 1 and Fig. 1). Thus, we examined the effect of SD on the protein level of IL1β. IL1β protein expression was assessed in the hypothalamus using the immunohistochemistry analysis. As shown in Fig. 2, the IL1β protein expression in the PVN of the hypothalamus was increased markedly in SD mice (16.53 ± 2.11% thresholded area) compared to that in the control mice (11.58 ± 1.44% thresholded area).

4. Discussion

In this study, we investigated the expression pattern of inflammatory genes by REM SD in the mice hypothalamus. Interestingly, the microarray analysis revealed that the expression of IL subfamily genes was changed by SD. The expression of IL1β, IL18, IL5, IL17E, and IL11RA1 genes increased in the hypothalamus of SD mice. In addition, we found that SD elevated not only the mRNA expression, but also the protein level of IL1β in the hypothalamus and particularly in the PVN.

IL subfamily genes play a major role in the regulation of immune responses, innate immunity, and inflammatory reactions [22]. In CNS, IL subfamily genes have a role in neuronal survival and differentiation, as well as in the balancing of neurodegeneration and neuroprotection [25]. Many studies have provided evidence that IL genes are also involved in sleep regulation. In particular, IL1β is well characterized for its role in sleep regulation [8]. Studies have shown that IL1β gene expression was increased in the brain regions including the hypothalamus, hippocampus, and cerebral cortex after SD [15,26]. In addition, injection of IL1β into the brain induced excess NREM sleep. Conversely, inhibition of IL1β using the anti-IL1β antibodies [19] and the IL1 receptor antagonist [27] inhibited sleep. The role of IL18 has been also demonstrated in sleep regulation. A previous study showed that IL18 injection in brain increased NREM sleep [11]. A positive association was also found between higher plasma IL18 level and poorer quality of sleep in peritoneal dialysis patients [29]. In our study, we found that REM SD increased not only IL1β and IL18, but also IL11RA1, IL17E, and IL5 in the mice hypothalamus. In particular, mRNA and protein expression of IL1β was markedly increased after SD. Thus, our finding, together with

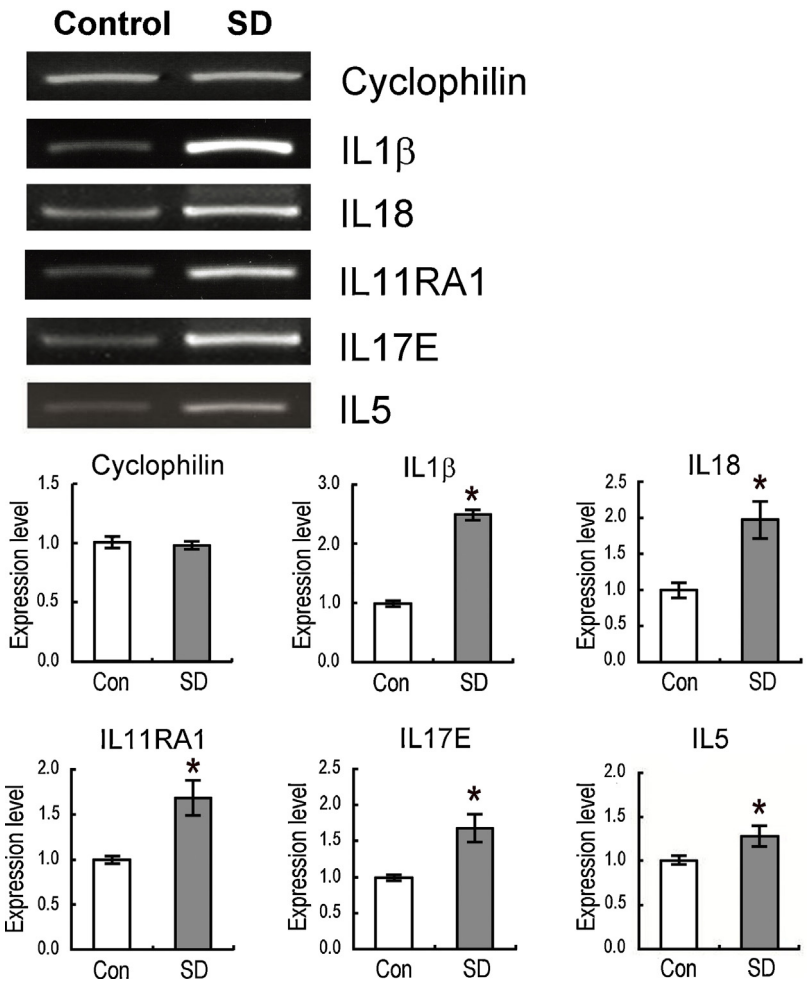

Fig. 1. Expression change of interleukin (IL) subfamily genes by REM SD in mice hypothalamus. For confirmation of microarray results, five IL genes, IL1β, IL18, IL11 receptor alpha chain 1 (IL11RA1), IL17E, and IL5 were analyzed by RT-PCR with total RNA from the hypothalamus of control and SD (72 h) mice (upper panel). For quantification of the RT-PCR result, expression level of each gene was measured as optical density (lower panel). Results are presented as mean ± S.E.M. (*$p < 0.05$, SD group versus control group). As an internal control, Cyclophilin was amplified. Three independent experiments were performed.

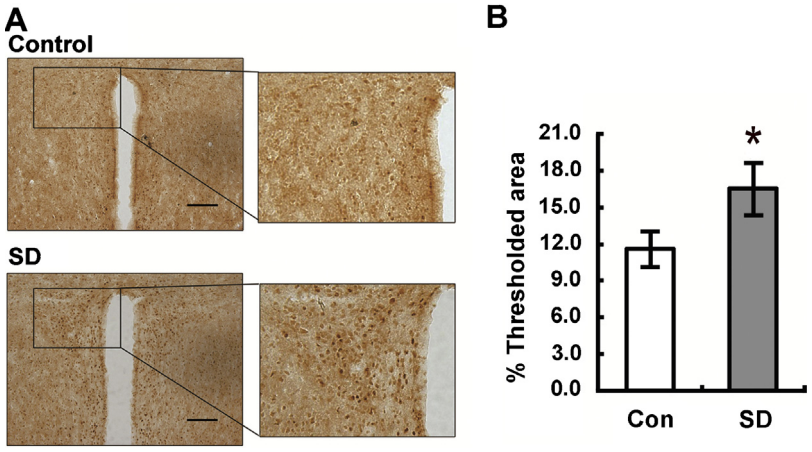

Fig. 2. Protein expression of interleukin 1 beta (IL1β) in the hypothalamus of mice with REM SD. IL1β protein expression was assessed in the hypothalamus of control and SD (72 h) mice using immunohistochemical staining (a). The immunoreactivity level of IL1β was measured quantitatively as percentage thresholded area in paraventricular nucleus (PVN) of the hypothalamus (b). Results are presented as mean ± S.E.M. (*$p < 0.05$, SD group versus control group). Three independent experiments were performed. Scale bar: 100 μm.

published observations, indicates that IL subfamily genes may play an important role in sleep regulation.

Interestingly, we found that the levels of IL subfamily genes such as IL11RA1, IL17E, and IL5 were increased in the hypothalamus by SD. This is the first finding that SD could affect levels of IL11RA1, IL17E, and IL5 genes. In the brain, the roles of IL11RA1, IL17E, and IL5 have not been elucidated, yet. However, several studies have reported that IL11, which specifically binds to IL11RA1 and their binding transmits IL11 signals [7], and IL5 have anti-inflammatory and neuroprotective effects [17,31]. Although IL17E appeared to be a pro-inflammatory cytokine inducing IL8 production [12], it could also affect level of anti-inflammatory cytokines such as IL5 and IL10 [20]. Indeed, overexpression of IL17E in mice elevated serum level of IL5 and gene expression of IL5 and IL10 in many tissues [20]. Although in our knowledge, there are not any reports about the involvement of IL11RA1, IL17E, and IL5 in sleep regulation or SD, considering previous studies, we speculated that anti-inflammatory cytokine genes (IL11RA1 and IL5) and cytokine gene regulating anti-inflammatory effect (IL17E) might be increased as a compensatory effect against increased expression of pro-inflammatory cytokine IL1β, and IL18 in SD. Further studies about the involvement of these IL subfamily genes in SD may be needed.

SD procedures using platform in water tank are affected by confounding variables, including stress associated with isolation, movement restriction, wetness and muscle fatigue. A previous study showed that plasma levels of corticosterone were elevated in rats of all groups placed on multiple large and small platforms and on single large and small platforms compared to rats remained in their home cages [23]. The corticosterone level was higher in rats placed on multiple platforms than rats placed on single platform despite of movement restriction and social isolation of rats by single platform, and this response was suggested to be attributed to social instability among rats coming from different cages [23]. Other study showed that plasma corticosterone levels were attenuated in social stable rats sleep-deprived on multiple platforms along with their peers compared to social instable SD rats [24]. However, the levels in social stable SD rats were still higher than social stable rats in home cages [24]. SD procedure used in our study could reduce stress such as movement restriction, social isolation or social instability compared to previously described single or multiple platform method. In addition, we assessed effect of SD through comparison between rats placed on small and large multiple platforms, not rats remained in their home cage. However, the effects by stress in our results that SD increased IL subfamily genes may not be completely excluded. Future studies on difference of stress responses, corticosterone levels, and their effects on IL gene expression between rats placed on small and large platforms along with their peers may help to further clarify these issues.

In conclusion, we found that REM SD could regulate the expression of IL subfamily genes, such as IL1β, IL18, IL5, IL17E, and IL11RA1, in the hypothalamus. In particular, REM SD strongly enhanced the expression of IL1β. Our findings suggest that IL subfamily genes, and in particular IL1β, might be involved in sleep regulation.

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2013.09.050.

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