Conditions that affect sleep alter the expression of molecules associated with synaptic plasticity

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The hypothesis that sleep serves a synaptic function was first proposed by Moruzzi (35). Although Moruzzi identified the level at which sleep could serve a function, it was presented within the context of a “local homeostasis” concept postulating that, during wakefulness, there is an accumulation of “wear and tear” in used neurons and that sleep serves to reverse these effects. In light of modern knowledge that neural use enhances synaptic efficacy (for review see Ref. 39), recent theories of sleep function have emphasized synaptic plasticity (10, 12, 23, 32). Although experimental support for these theories is limited, many of the molecules well characterized for their participation in sleep regulation (for review see Refs. 24 and 25) are also posited to play a role in synaptic plasticity. The list includes interleukin-1 (40, 49), tumor necrosis factor (1), nitric oxide (5, 18), adenosine (9), prostaglandins (3), nerve growth factor (29, 30), and nuclear factor-kB (39).

There is a relatively large literature, prompted by the Hebbian learning hypothesis (17), demonstrating changes in molecular markers of synaptic plasticity induced by long-term memory tasks (for review see Ref. 2). There is yet another literature, although somewhat smaller, demonstrating the influence of sleep on memory (42, 43). There is, however, little information concerning the influence of sleep loss or conditions that induce excess sleep on expression of molecules previously linked to synaptic plasticity. We investigated, therefore, the changes in mRNA associated with sleep deprivation and excess sleep of four molecules tightly linked to synaptic plasticity (for review see Ref. 2): activity-regulated cytoskeleton-associated protein (Arc), matrix metalloproteinase-9 (MMP-9), tissue plasminogen activator (tPA), and brain-derived neurotrophic factor (BDNF). BDNF is also directly linked to sleep regulation; injection of exogenous BDNF induces non-rapid eye movement sleep (NREMS) and rapid eye movement sleep (REMS) (27), and BDNF in the cerebral cortex increases during sleep deprivation (7, 38). We report here that sleep loss and a mild increase in ambient temperature (Tamb), a condition that enhances sleep in rats, affect the expression of Arc, MMP-9, tPA, and BDNF mRNAs.

METHODS

Sleep deprivation. Adult male Sprague-Dawley rats (320–350 g) were housed separately in a sound-attenuated environmental chamber (Hotpack 35-260) on a 12:12-h light-dark cycle (lights on at 0800) at 25°C. Rats were acclimated to the housing conditions for 10 days before they were killed. Four groups of rats (n = 8 in each group) were used. Group I rats were controls and were killed at 1600. Group II rats were deprived of all sleep by gentle handling as previously described (48) from 0800 to 1600. Samples comprising groups I and II were processed together. Group III rats were control rats for group IV and were killed at 1800. Group IV rats were sleep deprived for 8 h, allowed to recover for 2 h, and then killed at 1800. Rats in groups III and IV were processed separately from other groups. Rats were decapitated, and the...
cortex and hippocampus were rapidly dissected and quickly frozen in liquid N2. The time to remove, dissect, and freeze the tissue samples was <5 min.

Enhanced brain temperature. Adult male Sprague-Dawley rats (300–350 g) were used and housed separately as described above. Two groups of rats (n = 8 in each group) were used. Group V rats were control rats for group VI rats, and they were housed at 25°C; they were killed at 1400. Group VI rats were acclimated to 25°C housing conditions as were group V rats, but at 0800 their ambient temperature (Tamb) was increased to 28°C for the next 6 h. This mild increase in Tamb induces increases in NREMS and REMS (36, 47). Group VI rats were then killed at 1400. Rats in groups V and VI were killed, and brains were removed and dissected as described above.

RNA extraction. Total RNA was extracted by the method developed by Chomczynski and Sacchi (6) using combined phenol-chloroform and isopropanol precipitation. The RNA was dissolved in sterile water and checked using formaldehyde-containing agarose electrophoresis.

Table 1: Sense and antisense primers for Arc and tPA

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5′→3′)</th>
<th>Length of PCR Product, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arc</td>
<td>GAG TAG TCC TCC TCC AGC GCG CTC CCG TGG TCG TCC AGC ATC</td>
<td>336</td>
</tr>
<tr>
<td>BDNF</td>
<td>GGC TGC GCC CAT GAA AGA A</td>
<td>339</td>
</tr>
<tr>
<td>tPA</td>
<td>ACG CAC GAC GGA AGA AAC GGG CCG AAG CAG GGT GCT GTG CTT</td>
<td>408</td>
</tr>
<tr>
<td>MMP-9</td>
<td>CGT GGC CTA CTA GCG GAT CTA TG</td>
<td>592</td>
</tr>
<tr>
<td>CycA</td>
<td>CTT TGC AGA CGC CGC TGG TCG</td>
<td>474</td>
</tr>
</tbody>
</table>

Arc, activity-regulated cytoskeleton-associated protein; BDNF, brain-derived neurotrophic factor; tPA, tissue plasminogen activator; MMP-9, matrix metalloproteinase-9; CycA, cyclophilin A.
Cyclone PhosphorImager and OptiQuant analysis software (Packard Bioscience). Additional reagents were supplied by the RPA kit.

cDNA analysis. PCR products were excised and purified from agarose gels with use of a commercial gel extraction kit (QIAEX II, Santa Clarita, CA) following recommended protocols. Ten microliters (containing 1–1.5 μg of cDNA) of each gel-purified cDNA (Arc, CycA, BDNF, tPA, and MMP-9) were then subjected to restriction digestion with BanI and BanII (BioLabs, Beverly, MA) and SacI, SacI, and BamHI (Promega), respectively. All restriction digests included 20 U of enzyme in a total of 50 μl of reaction volume and were incubated for 2 h at 37°C. Ten microliters of each reaction were then loaded on 1.5% agarose gel containing ethidium bromide (0.5 μg/ml). Gels were run at 100 V for 60 min, and bands were photographed under ultraviolet light using a charge-coupled device camera (Gel Doc 1000 fluorescent gel bands were photographed under ultraviolet light using a charge-coupled device camera (Gel Doc 1000 fluorescent gel documentation system, Bio-Rad Laboratories). cDNA fragments obtained from each enzymatic digestion were of the correct size on the basis of the predicted amplified sequence (data not shown).

Statistical analysis. Using results from phosphorimaging data, we determined the ratio of the density of the target RNA, relative to that of the internal standard, expressed as data, we determined the ratio of the density of the target mRNA, relative to that of the internal standard, expressed as

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RESULTS

Effects of sleep deprivation. After 8 h of sleep deprivation, BDNF mRNA determined by RT-PCR increased by ~120% in the cortex (compare group I with group II in Table 2, Fig. 2). In contrast, sleep deprivation did not affect BDNF mRNA levels in the hippocampus (Table 2). With the use of a different method of analysis, i.e., RPA, similar results were obtained. Thus cortical levels of BDNF mRNA determined by RPA increased and hippocampal levels were not affected after sleep deprivation (Table 3, Fig. 3). After 2 h of recovery following 8 h of sleep deprivation, BDNF mRNA levels, determined by RT-PCR, were indistinguishable from corresponding control values in the cortex and hippocampus (compare group III with group IV in Table 2).

Sleep deprivation also enhanced the levels of Arc mRNA in the cortex whether determined by RT-PCR or real-time PCR. In contrast to the BDNF results, Arc mRNA increased in the hippocampus after sleep deprivation (compare group I with group II in Table 2). After 2 h of recovery, Arc mRNA levels in the cortex and hippocampus were similar to corresponding control values (compare group III with group IV in Table 1).

The effect of sleep loss on MMP-9 mRNA levels was in the opposite direction of that described for BDNF and Arc mRNAs. Thus, after 8 h of sleep deprivation, MMP-9 levels decreased ~35% in the cortex and hippocampus with RT-PCR or real-time PCR (compare group I with group II in Table 2). After 2 h of recovery following 8 h of sleep deprivation, MMP-9 mRNA levels were not significantly different from corresponding control values (compare group III with group IV in Table 2).

Table 2. Changes in brain of mRNA levels determined by RT-PCR or real-time PCR after sleep deprivation

<table>
<thead>
<tr>
<th>Group</th>
<th>BDNF</th>
<th>Arc</th>
<th>MMP-9</th>
<th>tPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0.57 ± 0.03</td>
<td>0.36 ± 0.05</td>
<td>1.04 ± 0.10</td>
<td>0.50 ± 0.04</td>
</tr>
<tr>
<td>II</td>
<td>1.27 ± 0.18*</td>
<td>1.06 ± 0.15*</td>
<td>0.68 ± 0.05*</td>
<td>0.67 ± 0.06*</td>
</tr>
<tr>
<td>III</td>
<td>0.62 ± 0.08</td>
<td>0.51 ± 0.14</td>
<td>0.51 ± 0.04</td>
<td>0.43 ± 0.09</td>
</tr>
<tr>
<td>IV</td>
<td>0.86 ± 0.12</td>
<td>0.51 ± 0.07</td>
<td>0.67 ± 0.03</td>
<td>0.68 ± 0.07</td>
</tr>
<tr>
<td>Hippocampus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0.56 ± 0.03</td>
<td>0.46 ± 0.05</td>
<td>1.33 ± 0.19</td>
<td>0.63 ± 0.07</td>
</tr>
<tr>
<td>II</td>
<td>0.61 ± 0.04</td>
<td>0.33 ± 0.11</td>
<td>1.14 ± 0.01</td>
<td>0.96 ± 0.13</td>
</tr>
<tr>
<td>III</td>
<td>0.64 ± 0.09</td>
<td>0.74 ± 0.08</td>
<td>0.77 ± 0.06</td>
<td>1.90 ± 0.28</td>
</tr>
<tr>
<td>IV</td>
<td>0.91 ± 0.11</td>
<td>0.92 ± 0.10*</td>
<td>0.69 ± 0.04</td>
<td>2.10 ± 0.14</td>
</tr>
</tbody>
</table>

Values are means ± SE of ratios of the radioactivity in the PCR product of interest to that in the band of the housekeeping control band; values in parentheses are means ± SE determined by real-time RT-PCR. *Significant difference from corresponding control, P < 0.05.
higher after sleep deprivation in the hippocampus, but this effect was not significant. tPA mRNA levels after 2 h of recovery following 8 h of sleep deprivation were similar in controls and experimental animals in the cortex and hippocampus.

Effects of mild increases in $T_{amb}$. After 6 h at 28°C, BDNF mRNA levels decreased significantly in the cortex (compare group V with group VI in Table 4; Fig. 2, right). Although BDNF mRNA levels were less in the hippocampus after 6 h at 28°C, they did not reach statistical significance. Arc mRNA levels determined by RT-PCR or real-time PCR also significantly decreased after 6 h at 28°C in the cortex. In contrast to BDNF mRNA, the mild increase in $T_{amb}$ also resulted in a significant decrease in Arc mRNA levels in the hippocampus (Table 4). MMP-9 levels were significantly increased in the cortex after treatment at 28°C $T_{amb}$ whether determined by RT-PCR or real-time PCR. In the hippocampus, MMP-9 values increased almost threefold as determined by RT-PCR (not significant) and about twofold as determined by real-time PCR (significant). tPA mRNA levels, on the other hand, significantly decreased in the cortex after 6 h of treatment at 28°C $T_{amb}$. tPA mRNA levels in the hippocampus were not affected by the $T_{amb}$ treatment.

DISCUSSION

The results presented here are consistent with previous related work. Thus BDNF mRNA levels were previously reported to increase after sleep deprivation (7, 8, 38). Furthermore, RT-PCR-derived data were confirmed by RPA- or real-time PCR-derived data. The decreases in MMP-9 mRNA after sleep deprivation are similar to decreases in MMP-9 enzymatic activity (J. W. Harding and J. Wright, unpublished observations) after 8 h of sleep deprivation following learning trials in a Morris water maze. Finally, Cerelli and Tononi (7, 8) also reported that Arc mRNA and protein levels are higher after sleep deprivation than after sleep. The present results extend these previous findings by providing a systematic comparison of the expression of mRNA of four synaptic plasticity-associated molecules after sleep deprivation and after 2 h of recovery following sleep deprivation. We also determined what happens to the expression of these molecules after an increase in $T_{amb}$, a condition that enhances sleep (36).

Sleep deprivation is used often in sleep research to investigate the homeostatic regulation of sleep; after sleep deprivation, sleep rebound occurs. This rebound sleep is thought to result from the enhanced production during sleep deprivation of sleep-promoting and sleep-regulatory substances. For instance, the transfer of cerebrospinal fluid from sleep-deprived to non-sleep-deprived animals induces increases in sleep in the recipient (37). As indicated in the introduction, many of the well-characterized sleep-regulatory substances are also involved in synaptic plasticity. BDNF may be one of these sleep-regulatory substances, although evidence for its role in sleep regulation is limited to the findings that if it is injected, it induces sleep (27) and its levels in the cortex increase during sleep deprivation (7, 38). However, there is extensive evidence for its role in neurite growth and synaptic plasticity (for review see Ref. 34). Whether the other substances measured in this study, Arc, MMP-9, or tPA, have any role in sleep regulation has not been investigated. However, given the extensive evidence for their involvement in synaptic plasticity (for review see Ref. 2), that their levels are affected by sleep loss and excess sleep could be indicative of their involvement in sleep function.

Many studies in several species have confirmed the finding that mild increases in $T_{amb}$ are associated with increases in NREMS and REMS (36). REMS is particularly sensitive to $T_{amb}$; maximum duration of REMS occurs at thermoneutral $T_{amb}$ (47). Electroencephalographic (EEG) and electromyographic recordings from our animals would have been necessary to determine the amount of sleep they had before death. However, we chose not to implant EEG and electromyographic electrodes, because wounds induce long-term upregulation of cytokines, which, in turn, can affect expression

Table 3. Levels of BDNF mRNA determined by RINase protection assay in cortex and hippocampus after 8 h of sleep deprivation

<table>
<thead>
<tr>
<th>Group</th>
<th>BDNF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cortex</td>
</tr>
<tr>
<td>I</td>
<td>0.25 ± 0.01</td>
</tr>
<tr>
<td>II</td>
<td>0.48 ± 0.05*</td>
</tr>
</tbody>
</table>

Values are means ± SE as described in Table 2 footnote. *Significant difference from group I, $P < 0.05$. 

J. W. Harding and J. Wright, unpublished observations.)
of some of the molecules we measured, e.g., MMP-9 (50). Furthermore, the presence of EEG electrodes on the surface of the dura could lead to inflammation; the cytokine-chemokine cascade associated with inflammatory responses would likely affect expression of a host of molecules, including those we measured. Finally, the observation that mild increases in T_{amb} enhance sleep is one of the most reliable, reproducible results in sleep research. The advantage of using T_{amb} to manipulate sleep is that it does not involve handling the animals, nor is it a stressor.

A fundamental hypothesis forming the basis for Hebbian learning is that synaptic activation enhances the efficacy of transmission in those synapses during subsequent activation. The enhanced efficacy is thought to result from molecules, the upregulation and release of which are dependent on synaptic activation (for review see Refs. 16 and 28). Arc is an immediate-early gene (30). Arc protein interacts with cytoskeletal protein and likely modifies structural protein. There is strong evidence that Arc mRNA and protein are tightly coupled to the activity state of neurons (28, 30). Within 30 min of neural activation, Arc mRNA is found throughout the dendrites of granule cell neurons (16, 28, 30, 44). Translation of Arc mRNA in specific activated dendrite spines is likely targeted via the assembly of the translational complex, which is induced by activation of metabotropic glutamate receptors (45). This represents a mechanism by which the initiation of Hebbian learning by waking activities can be targeted to the specific circuits activated. Furthermore, caffeine, a well-known inhibitor of sleep, induces Arc expression via a step involving the adenosine A1 receptor (11). That prolonged wakefulness (sleep loss) and excess sleep alter Arc mRNA expression may reflect a disturbance of this mechanism.

A well-recognized action of tPA is to catalyze the cleavage of plasminogen to the active protease plasmin. Plasmin is an important activator of latent MMPs. As such, we anticipated that increased tPA mRNA would translate into increased MMP-9 mRNA. That it did not is likely due to the multiple roles of tPA. Furthermore, we measured MMP-9 mRNA, not protein activity; tPA and plasmin may not affect MMP-9 mRNA expression. Nonetheless, tPA is recognized as a participant in neural remodeling (15), and our results indicate that its mRNA is affected by sleep loss and excess sleep.

The extracellular matrix is of central importance to nervous tissue structure and function. The composition of the extracellular matrix is in a dynamic flux that

![Image of RNase protection assay of BDNF mRNA](image-url)

**Fig. 3.** RNase protection assay of BDNF mRNA. BDNF mRNA levels in the cerebral cortex (CT) increased after 8 h of sleep deprivation (SD) compared with controls (C). BDNF mRNA levels in the hippocampus (HC) were not affected by sleep deprivation. Samples were prepared and visualized as described in METHODS; 1 sample per experimental group is shown. A total of 8 samples from 8 rats were analyzed (see Table 3 for results).

### Table 4. Changes in cerebral cortex and hippocampus of mRNA levels determined by RT-PCR or real-time PCR after mild increases in ambient temperature

<table>
<thead>
<tr>
<th>Group</th>
<th>BDNF</th>
<th>Arc</th>
<th>MMP-9</th>
<th>tPA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cortex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>0.46 ± 0.05</td>
<td>0.51 ± 0.06</td>
<td>0.63 ± 0.04</td>
<td>0.43 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>(1.16 ± 0.25)</td>
<td>(1.02 ± 0.07)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>0.17 ± 0.01*</td>
<td>0.20 ± 0.03*</td>
<td>0.76 ± 0.003*</td>
<td>0.21 ± 0.02*</td>
</tr>
<tr>
<td></td>
<td>(0.57 ± 0.07*)</td>
<td>(1.31 ± 0.11*)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hippocampus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>0.89 ± 0.10</td>
<td>0.91 ± 0.11</td>
<td>1.02 ± 0.10</td>
<td>0.72 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>(1.09 ± 0.12)</td>
<td>(1.19 ± 0.21)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>0.75 ± 0.05</td>
<td>0.42 ± 0.03*</td>
<td>2.94 ± 1.4</td>
<td>0.57 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>(0.73 ± 0.05*)</td>
<td>(2.64 ± 0.61*)</td>
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</tbody>
</table>

Values are means ± SE as described in Table 2 footnote; values in parentheses are means ± SE as determined by real-time RT-PCR.

*Significant difference from group V, P < 0.05.
represents a balance between synthesis of components and degradation, which is mediated by MMPs. The involvement of MMP-9 in neural plasticity has been suggested by several studies (for review see Ref. 7). MMP-9 expression is evident at the neuromuscular junction and is enhanced after nerve crush at a time when repair and remodeling are occurring (22). Second, human gliomas in culture exhibit an inverse relationship between MMP-9 and neural cell adhesion molecule (31), thereby suggesting that perhaps the MMP-9 is degrading neural cell adhesion molecule with the purpose of decreasing cell adhesiveness and augmenting the potential for structural reorganization. Finally, expression of MMP-9 occurs in the rat hippocampus during the consolidation phase of spatial learning (19). Our result that MMP-9 mRNA is downregulated by sleep deprivation is consistent with the notion that MMP-9 plays a role in learning, since sleep loss attenuates memory consolidation (42, 43).

Perspectives

There is a growing body of evidence suggesting that sleep is not a uniform process of the entire brain but is, instead, a local property of highly interconnected neurons and that the local state shifts are dependent on prior neural activity, rather than duration of wakefulness (23). Initially predicted from theoretical considerations (23, 25), it was subsequently shown that patterns of brain activity during sleep are dependent on prior wakefulness activity. For example, Kattler et al. (21) demonstrated that mechanical stimulation of one hand enhances EEG slow-wave activity [a measure of the depth of sleep (37)] in the contralateral somatosensory cortex during subsequent sleep. Drummond et al. (13), using functional magnetic resonance imaging, reached the conclusion that the localized effects of sleep deprivation are dependent, in part, on the specific cognitive task performed in prior wakefulness. These and other data suggest that sleep is targeted and, similar to synaptic efficacy, dependent on prior synaptic activation. Our data are consistent with this notion to the extent that manipulations that affect sleep, sleep deprivation and mild increases in Tamb, affected expression of molecules associated with synaptic plasticity.

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