Effects of sleep deprivation on behaviors and abnormal hippocampal BDNF/miR-10B expression in rats with chronic stress depression

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Abstract: Being sleep-deprived can relieve the depressed emotions in rats, but the underlying mechanisms remain unknown. In this study, male rats were divided into 3 groups: normal control (NC), chronic unpredictable stress (CUPS) and sleep-deprived (SD). All of the groups were examined using the sucrose consumption test and the open field test. The sucrose consumption test and the open field test were performed for all three groups. The BDNF and miR-10B expressions were examined using real-time PCR and the level of BDNF was discovered by western blotting. In the sucrose consumption test and the open field test, the CUPS rats consumed less sucrose and got fewer score than the NC rats, however the SD rats consumed significantly more sucrose and received higher scores than the CUPS rats. Both the expression of BDNF and the protein levels in the CUPS group was significantly lower than in the NC group. Also, the CUPS group also showed a higher miR-10B expression than the NC group. However, the SD group demonstrated higher BDNF expression and lower miR-10B expression when compared with the CUPS group. Further investigation demonstrated that the BDNF is the direct target gene of miR-10B and BDNF expression, which is negatively correlated with the expression of miR-10B. In the sucrose consumption test, BDNF expression is positively correlated with the sucrose preference rate whereas miR-10B has an opposing correlation. Moreover, the open field test demonstrated that BDNF expression is positively correlated with the scores and the miR-10B expression is negatively correlated. These results indicate that sleep deprivation is closely linked with the downregulation of miR-10B and possibly the upregulation of BDNF in the hippocampus in the CUPS rats.

Keywords: Sleep deprivation, MicroRNA-10b, BDNF
Sleep deprivation in chronic stress depression

Table 1. Rat behavior tests among normal (NC) group, depression (CUPS) and sleep deprivation (SD) groups (M ± SD, n=10 for each group)

<table>
<thead>
<tr>
<th>groups</th>
<th>Sucrose consumption test</th>
<th>Open field experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal group</td>
<td>0.91±0.22</td>
<td>98.63±7.79</td>
</tr>
<tr>
<td>Depression group</td>
<td>0.23±0.09</td>
<td>34.57±5.54</td>
</tr>
<tr>
<td>Sleep deprivation</td>
<td>0.76±0.15</td>
<td>82.32±6.38</td>
</tr>
</tbody>
</table>

Compared with NC group, *, P < 0.05; compared with CUPS group, †, P < 0.05.

The age of 3 months were provided by the Experimental Animal Co. Lake Hayes. And randomly divided into three groups: normal control (NC, n=10), chronic unpredictable stress (CUPS, n=10) and sleep-deprived (SD, n=10). Rats in control group received standard husbandry care. Rats in the CUPS and SD groups were subject to chronic unpredictable stress for 3 weeks. And SD group was treated with a Sleep deprivation tank.

Chronic unpredictable stress (CUPS)

The CUPS paradigm was performed following a previously established protocol [21] with minor modifications. In summary, rats were randomly exposed to one of the following stressors once a day for 3 weeks: water deprivation for 24 hrs, food deprivation for 24 hrs, swimming in cold water (4°C) for 5 min, exposure to an elevated open platform (10 cm × 10 cm, 160 cm in height) for 2 hrs [22], restraint stress for 2 hrs [23] or electric foot shock for 20 s (800 mA, 1-s duration, average 1 shock/10 s). Stress was given at different times of the day to establish unpredictability.

Sleep-deprived (SD)

After performed Chronic Unpredictable Stress for 3 weeks, the SD rats were put in a sleep deprivation tank: 30 cm × 30 cm × 30 cm, in a straight live 6 cm, 8 cm high platform. The platform around the periphery was filled with water; the water temperature was 20°C. The distance of platform from the surface was 1 cm and the tank temperature controlled at 18-22°C for 48 hours.

Sucrose consumption test

Rats had access to both tap water and a 1% sucrose solution in their habitat. Fluid consumption was recorded by reweighing pre-weighed bottles of test solution. Bottles were counterbalanced across left and right sides of the cages throughout the experiment. The percent preference (PP) for sucrose was calculated by determining the percentage of total fluid consumption accounted for by ingestion of the 1% sucrose solution.

Open field test

Open field test was carried out as previously described [24]. The open field arena was made...
of an open rectangular plastic box (100 cm × 100 cm × 30 cm) with 25 squares (20 cm × 20 cm) painted on the floor. The 25 squares included 16 peripheral squares and 9 central squares. During the test, the rats were placed individually in the middle of the field and were allowed to explore the area freely for 10 minutes. The activities of the rats were monitored using an overhanging camera that was linked to a computer. Ethovision 3.0 (Noldus, The Netherlands) was used to track the behaviors of the rats. Such behaviors included the total distance a...
rat moved in the arena, distance a rat moved in the central squares (1 point/square), the number of vertical activity (1 point/square), and the number of fecal pellets present in the arena (0.5 point/pellet) during the 10 minutes were recorded. The arena was cleaned with 75% alcohol between trials to ensure that the imprint of previous rats did not affect a rat’s behavior.

Sample collection

All rats were sacrificed 24 hrs after the last behavioral test. Rats were anesthetized with intraperitoneal injections of 1% Pentobarbital (20 mg/kg body weight). Brains were rapidly removed from the skull, and hippocampus tissues were immediately sectioned according to the anatomical atlas of Paxinos and Watson [24]. Tissues were snap frozen in liquid nitrogen and kept at -80°C until use.

Real-time reverse transcription quantitative PCR

Quantitative RT-PCR was used to detect BDNF mRNA and miR-10B microRNA in hippocampus tissues. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription of BDNF and miR-10B was performed using Revert Aid First Strand cDNA synthesis kit (MBI Fermentas, Burlington, Canada) and One Step PrimeScript® miRNAcDNA Synthesis Kit (Perfect Real Time, TaKaTa, Japan) following the manufacturer’s protocol respectively. Real-time quantitative PCR was performed using ABI Prism® 7900HT (Perkin-Elmer, Applied Biosystems). The primers used for BDNF, miR-10B, β-actin, and U6 were respectively: BDNF (5’-aagtgcctttggagcctcct-3’, 5’-gctaatactgtcacacacgc-3’), miR-10B (5’-acactactgtcacacacgc-3’), miR-10B (5’-acactactgtcacacacgc-3’), miR-10B (5’-acactactgtcacacacgc-3’), and U6 (5’-ctcagttgtgccctgtgaaccgaatt-3’).
gttcctgtgga-3'), β-actin (5'-GGAGATTACTGCCCT-GGCTCCTA-3', 5'-GACTCATCGTACTCCTGCTTG-C TG-3') and U6 (5'-ctcctggccagcaca-3', 5'-aagcttcagaaattgc-3')

The data were analyzed by comparing miR-10B and BDNF mRNA levels with U6 and β-actin level, respectively for each rat and then subjected to statistical analysis. All qRT-PCR reactions were performed thrice.

**Western blot**

Hippocampus tissues were harvested as described above. Western blots were performed as previously described [26]. The anti-BDNF (1:500 dilution), anti-β-actin (1:1000 dilution), and HRP-conjugated second antibodies (1:2000 dilution) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cloning of 3UTRs into PsiCHECK2 luciferase expression plasmid**

BDNF-3'UTR sequence was PCR amplified from rat genomic DNA (primers: 5'-aaaagcggccgctg-gatttatgttgtatagatt-3' and 5'-aaaactcgagcactatatagaacctgtat-3'). PCR fragment was ligated into pGem-T easy vector (Promega) according to the guidelines of the manufacturer and further subcloned into a single NotI site at the 3 end of luciferase in the PsiCHECK2 reporter plasmid (Promega). Cloning orientation was verified by diagnostic cuts followed by sequencing. A mutated form of BDNF-3'UTR lacking all 7 bases of miR-10B conserved seed-match sequence was established by gene rating two partially complementary PCR fragments, used as templates for ligation PCR (primers: 5’-ctatttgataatatatacatagccaaattattcag-3 and 5’-ctgattaatatatacatagcgtgtatatatacaaaatag-3, each used with one of the aforementioned primers). The mutated PCR fragment lacks all 7 bases of miR-10B conserved seed-match sequence and includes a new BamHI restriction site to facilitate diagnostics. It was also cloned as described for the intact form.

**Transfections and luciferase assay in HEK293T cells**

Cells were grown on poly-L-lysine in 24-well format to an 80-85% confluency and transfected using Lipofectamine with the following plasmids: 2 μg of psiCHECK-2-3'UTR plasmids and 500 ng of miR-10B mimicsor the inhibitor of miR-10B (Genepharma, Shanghai, China). At 48 h after transfection, cells were lysed, and luciferase reporter activity was assayed as described previously [27]. Renilla luciferase values were normalized to control firefly luciferase levels (transcribed from the same vector but not affected by 3'UTR tested) and averaged across three -well repetitions per condition. Data presented are an average of the three experiments.

**Statistical analysis**

Data was presented as mean and standard error of the mean, and the statistical package for the Statistical Product and Service Solutions (SPSS) 17.0 was used to analyze the data. One-way analysis of variance (ANOVA) or Kruskal-Wallis LSD-test or Nemenyi-test was used for analyzing the difference between groups. Correlation between BDNF, miR-10B expression, and behavioral indexes were analyzed using Pearson correlation test. A P < 0.05 was considered statistically significant.

**Results**

**Effect of CUPS and SD on the behaviors of adult rats**

Behavioral data obtained from sucrose consumption test and open field test are presented in Table 1. The Sucrose consumption test showed significant differences in sucrose consumption between CUPS group and NC group (P < 0.05). And the SD rats consumed more sucrose than CUPS rats (P < 0.05), but not than NC rats. As shown in Table 1. The score of the open field arena was significantly different between CUPS group and NC groups (P < 0.05), while the CUPS rats had less score than NC rats. And the SD group has high score than CUPS group (P < 0.05) while exhibited no difference compared to NC group (P < 0.05).

**Effect of CUPS and SD on BDNF mRNA, BDNF protein, and miR-10B mRNA levels in the hippocampus of adult rats**

It is believed that the onset of depression involves the BNDF transcribed from the 5’exon VI and 3’common exon. The reason being is that these particular transcripts are not only abundant in the hippocampus but also play an important role in both neuronal survival and synaptic plasticity [28-30]. As expected, significant differences in BDNF mRNA expression were observed in the hippocampus between
CUPS group and NC group ($P < 0.05$), CUPS rats showed significantly lower BDNF mRNA expression than NC group (Figure 1A). Meanwhile, significant difference in BDNF mRNA expression was observed in the hippocampus between SD group and CUPS group ($P < 0.05$), SD rats showed significantly higher BDNF mRNA expression than CUPS rats (Figure 1A). As shown in Figure 1B, significant differences in BDNF protein expression were observed in the hippocampus between CUPS group and NC group, CUPS rats showed significantly lower BDNF protein expression than NC rats. However, the SD group showed significantly higher BDNF protein expression than CUPS group. Significant differences in miR-10B mRNA expression were observed in the hippocampus between CUPS group and NC group ($P < 0.05$), CUPS rats showed significantly higher miR-10B mRNA expression than NC group (Figure 1C). While SD rats showed significantly lower miR-10B mRNA expression than NC rats (Figure 1C).

**BDNF is the direct target gene of miR-10B**

We found that the expression level of BDNF decreased as the expression of miR-10B increased in all of the examples. In addition, Pearson correlation analysis revealed that BDNF mRNA expression negatively correlated with the expression of miR-10B in all of the rats (Figure 2A, $r=-0.773$, $P < 0.001$). With a widely used open-access software of target scan [31], we found that miR-10B targets the 3'UTR of BDNF with a high conservation (Figure 2A), which has not been reported previously. In order to validate our prediction, a luciferase reporter assay was further performed. As shown in Figure 2B, miR-10B co-transfection in 293T cells dramatically increased the relative luciferase activity of the vector encoding the wt-BDNF 3'UTR, but not the vector with mut-BDNF 3'UTR. Moreover, the relative luciferase activity remained as low as in the control group in the presence of anti-miR-10B inhibitor (Figure 2B). These data showed that miR-10B was BDNF's upstream regulator and targeted TSGA10 at its 3'UTR.

**Correlation of BDNF mRNA/miR-10B expression and behavioral indexes of rats**

We used the Pearson correlation analysis to confirm the correlation of the BDNF mRNA/miR-10B expression and the behavioral indexes of rats, and we found that BDNF mRNA expression positively correlated with the sucrose preference rate ($r=0.952$, $P < 0.001$) in the sucrose consumption test (Figure 3A) and the total scores in the open field test ($r=0.949$, $P < 0.001$, Figure 3B). MiR-10B expression negatively correlated with the sucrose preference rate ($r=-0.922$, $P < 0.001$) in the sucrose consumption test (Figure 3C) and the total scores in the open field test ($r=-0.952$, $P < 0.001$, Figure 3D).

**Discussion**

The direct relationship between neurobiological anomalies and stress-induced depressive behaviors cannot be verified in humans due to ethical limitations. Therefore, animal models are currently the ideal tools to elucidate the biological basis of stress-induced depressive behaviors. In this study, the up regulation of miR-10B and down regulation of BDNF expression was shown to be involved in all rat behaviors.

BDNF is an important member of the neurotrophin family of growth factors, which induces stress in both animal models [32] and human psychopathologies, including major depression, or posttraumatic stress disorder [33, 34]. Our results showed that the BDNF levels in CUPS group with Chronic Unpredictable Stress were significantly decreased compared with controls, and the SD group was significantly increased compared with CUPS group. Surprisingly, the rats in CUPS group included anhedonia and a lack of motivation in the Sucrose Consumption Test and the Open Field Test, while the SD rats showed less anxiety. This suggests that the expression of BDNF level is positively correlated with the depression, as reported in the Previous studies [35, 36]. In another report, BDNF over activity in depressive patients could be important, as it has been suggested that BDNF over activity plays a key role in the pathogenesis of the manic state [37]. However, in this study, the BDNF in rats with sleep-deprivation significantly increased compared with CUPS group, yet was still lower than NC group. In other words, sleep-deprived just rescue the expression of BDNF partly, but rapidly.

In this study, we also identified an abnormal expression of miR-10B which had been reported in some cancers, such as gastric cancer, oral cancer, and glioma [38-42]. The expres-
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Expression of miR-10B is negative correlation with BDNF in the hippocampus, and the BDNF is the direct target gene of miR-10B is an important finding in our study. In the two behavioral experiments, the rats which had the high miR-10B, had the least score in the open field test and had little consumption in the sucrose consumption test. This suggests that the expression of miR-10B is negative correlation with the behaviors. It is reasonable to believe that miR-10B may play a crucial role in the development of depression via simultaneously modulating the expression of BDNF in the hippocampus, although the involvement of miR-10B in the fine regulation of BDNF expression, essential for the adjustment of the behaviors and physiological conditions, is far less understood, this finding may lead to the development of novel treatments for anxiety disorders and depression and other stress-related psychiatric and physiological conditions.

Our study shows that the sleep deprivation is significantly associated with down regulation of miR-10B and possibly subsequent up regulation of BDNF in hippocampus in the chronic unpredictable stress rats. Our results further show the BDNF is the direct target gene of miR-10B. miR-10B may play an important role in anxiety disorders.

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Disclosure of conflict of interest
None.

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