Phosphodiesterase 10A inhibition attenuates sleep deprivation-induced deficits in long-term fear memory

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ABSTRACT

Sleep, particularly rapid eye movement (REM) sleep, is implicated in the consolidation of emotional memories. In the present study, we investigated the protective effects of a phosphodiesterase 10A (PDE10A) inhibitor MP-10 on deficits in long-term fear memory induced by REM sleep deprivation (REM-SD). REM-SD caused deficits in long-term fear memory, however, MP-10 administration ameliorated the deleterious effects of REM-SD on long term fear memory. Brain-derived neurotrophic factor (BDNF) and phosphorylated cAMP response element-binding protein (pCREB) were altered in specific brain regions associated with learning and memory in REM-SD rats. Accordingly, REM-SD caused a significant decrease of pCREB in hippocampus and striatum and a significant decrease of BDNF in the hippocampus, striatum and amygdala, however, MP-10 reversed the effects of REM-SD in a dose-dependent manner. Our findings suggest that REM-SD disrupts the consolidation of long-term fear memory and that administration of MP-10 protects the REM-SD-induced deficits in fear memory, which may be due to the MP-10-induced expression of BDNF in the hippocampus, striatum and amygdala, and phosphorylation of CREB in the hippocampus and striatum.

KEYWORDS: REM sleep deprivation, fear memory, phosphodiesterase-10

Highlights:

MP-10 ameliorates sleep deprivation-induced long term fear memory deficit

REM-SD causes a decline of pCREB and BDNF in hippocampus and striatum

Recovery of pCREB and BDNF levels by MP-10 benefits long term fear memory

1. Introduction

Sleep is composed of two prominent phases: rapid eye movement (REM) sleep or paradoxical (PS) sleep and non-rapid eye movement (NREM) sleep. Accumulating evidence suggests that REM sleep plays an important role in learning and memory [1-3]. In humans, REM sleep is known to facilitate long-term consolidation of visual discrimination tasks and emotional memories [4-6]. In experimental animals, post-learning REM sleep promotes the consolidation of newly acquired memories for long term storage [7-10]. It has been shown that post-learning REM-sleep deprivation (REM-SD) impairs spatial and environmental fear memory formation [11-13].

Memory consolidation is a process by which temporary short-term memory (STM) is stabilized into a persistent long-term memory (LTM). Neuronal plasticity plays a critical role in mediating the conversion of STM to LTM. Plasticity requires the activation of transcription factors for target gene transcription and protein translation [14-16]. Among the various transcription factors, cyclic AMP responsive element binding protein (CREB) is particularly important in memory consolidation [17-19]. Interestingly, some studies have reported that the cAMP-PKA-CREB pathway is sensitive to SD [20-23].

Phosphodiesterase 10A (PDE10A) is an isoform of the cAMP/cGMP phosphodiesterase family, which is highly expressed in the striatal complex, but expressed at lower levels in the hippocampal pyramidal cell layer, dentate granule cell layer, and throughout the cortex and cerebellar granule cell layer [24, 25]. Although PDE10A can simultaneously catalyze both cAMP and cGMP, it hydrolyzes cAMP at a rate that is approximately 60-fold higher than cGMP [26-29]. Therefore, inhibition of PDE10A leads to higher levels of cAMP than cGMP. PDE10 inhibitors have shown potential for the treatment of cognitive dysfunction, particularly social memory and executive function [30]. Interestingly, inhibition of PDE10A using the inhibitor TP-10 (2-[4-(1-methyl-4-pyridin-4-yl-1H-pyrazol-3-yl)-phenoxy]methyl]-quinoline succinic acid) increases dopamine turnover in the striatum and decreases amphetamine-stimulated locomotor activity [31], which has an effect on positive symptoms of schizophrenia. TP-10 increases phosphorylation of GluR1 and reverses the deficits induced by the NMDA receptor antagonist MK-801, which may contribute to the effects of TP-10 on the negative symptoms and cognitive dysfunction of schizophrenia [32, 33]. TP-10 also reverses scopolamine- and MK-801-induced memory deficits [34]. Recently, we reported that inhibition of PDE10A by MP-10 attenuates morphine-induced conditioned place preference [35]. Therefore, these studies indicate that PDE10A plays a role in the regulation of memory and emotion.

In the present study, we examined the effects of MP-10 (2-[4-(1-methyl-4-pyridin-4-yl-1H-pyrazol-3-yl)-phenoxy]methyl]-quinoline succinic acid), which is an inhibitor of PDE10A, on REM-SD-induced impairments in long-term fear memory. MP-10 administration effectively ameliorated the deleterious effects of REM-SD on long-term fear memory. Moreover, the levels of brain-derived neurotrophic factor (BDNF) and phosphorylated CREB were increased in the brain regions associated with learning and memory in animals after MP-10 administration, which suggests that MP-10 potentially reverses deficits in long-term fear memory by increasing the levels of pCREB and BDNF in specific brain regions. Therefore, our study provides evidence that PDE10A inhibitor has a potential therapeutic benefits for sleep-related memory deficits.

2. Methods and materials

2.1. Animals

Adult male Sprague-Dawley rats weighing between 200 and 250 g were used in this study. The animals were purchased from Shanghai Laboratory Animal Co., Ltd. (Shanghai, China) and habituated for at least 1 week before the start of the experiments. All animals were housed under standard laboratory conditions (i.e., 12/12 h light-dark cycle with lights on at 07:00 A.M, 22~24°C ambient temperature, and food and water ad libitum). All behavioral experiments were conducted during the animal's light cycle. The animal protocols were approved by the Institutional Animal Care and Use Committees of Soochow University and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Reagents and treatments

MP-10 was purchased from Shanghai PharmaResources Inc. (Shanghai, China). Dimethylsulfoxide (DMSO) was purchased from Amresco (USA). Cremophor EL was obtained from Sigma-Aldrich (USA). Tween 80 was purchased from Hua Yu (WUXI) Pharmaceutical Co., Ltd. (China). MP-10 was dissolved in a dissolvent containing 5% dimethylsulfoxide, 5% Cremophor EL, 5% Tween 80 and 85% saline. For the control treatment, the dissolvent contained the same composition but without the MP-10. MP-10 at concentrations of 0.25, 0.5 or 1.0 mg/ml was administered by intraperitoneal injection (2 ml/kg). Fear-conditioning training was conducted 30 min after MP-10 treatment.

2.3. Apparatus

Startle response, shock reactivity and fear-potentiated startle were measured using the Startle Reflex System and Advanced Startle Software (Med Associates Inc. USA). Animals were trained and tested in four identical 60×35×35 cm ventilated sound-attenuating startle chambers. A Plexiglas cage (16.5×6.0×5.0 cm) was suspended within a PVC frame, which was firmly fixed to a response platform by four thumbscrews in the startle chamber. The floor of the cage consisted of six 0.5 cm diameter stainless steel bars spaced 0.6 cm apart. A loudspeaker located behind the Plexiglas cage delivered the acoustic startle stimulus (105 dB; 20 ms burst of white noise, WN). Background noise (65 dB) existed throughout the experiments. The startle response was recorded after the onset of the WN. The peak amplitude of the response was used to calculate the startle response, shock reactivity and fear-potentiated startle (FPS).

2.4. Behavioral procedures

The behavioral procedure was comprised of the following phases: acclimation, baseline startle test, fear-conditioning training (learning), paradoxical sleep deprivation, and fear-potentiated startle test.

2.4.1. Acclimation

To acclimate the animals to the apparatus and startle stimuli, the rats were placed into the test chambers for 10 min and then returned to their home cages. This procedure was repeated

for three acclimation days.

2.4.2. Baseline startle test

To measure levels of baseline startle, the rats were subjected to 2 days of baseline startle testing prior to training. During a 5-min acclimatization period, the rats received no stimuli, with the exception of background noise. The initial WN (105 dB; 20 ms) was then presented three times with a 30 s inter-stimulus interval (ISI) to obtain a stable baseline acoustic startle response (ASR). Thereafter, the rats were presented with 48 WN stimuli (twelve at each intensity 90, 100, 110 and 120 dB). The various stimulus intensities were presented in a semi-random order with a 45 s ISI. Rats were matched into groups according to the averaged startle amplitude on the second day to obtain a similar overall mean startle amplitude [36, 37].

2.4.3. Fear-conditioning training (learning)

Twenty-four hours after the baseline startle test, the rats were administered MP-10 or the control dissolvent. The rats were placed back in the test chambers 30 min after drug treatment. After a 5-min acclimation, 30 pure tone-foot shock pairings were presented. Foot shocks (unconditioned stimuli, US) were delivered immediately after the pure tone (conditioned stimulus, CS). The CS duration was 3000 ms. The foot shock was sustained for 250 ms and the strength was 0.6 mA. The average inter-trial interval was 2 min (range = 1~3 min).

2.4.4. Paradoxical sleep deprivation

Rats were selectively deprived of REM sleep for 6 h using the modified multiple platform method (MMPM) [38] immediately after fear-conditioning training. This method results in a near total loss of REM sleep and a partial loss of NREM sleep [39, 40]. Small platforms (diameter 6.3 cm, height 10 cm) were used for both REM-SD and REM-SD+MP-10 rats, and larger platforms (diameter 12 cm, height 10 cm) were used for the large platform (LP) rats as a control group. Normal control (CON) rats were placed in their home cage after training. The platforms were spaced 7~8 cm apart. The water temperature was maintained at $22\pm 1^{\circ}\text{C}$. Food and water was available *ad libitum* during the experiments. When rats on the small platforms entered REM sleep, they lost muscle tone and fell into water, which caused them to awaken. However, the rats in the LP condition could maintain normal sleep because the platforms were large enough to accommodate their body.

2.4.5. Fear-potentiated startle (FPS) test

All rats were given a fear-potentiated startle test 24 h after fear-conditioning training. The animals were placed in the test chambers followed by a 5 min habituation period. The initial WN (120 dB; 20 ms) was then presented three times with a 30 s ISI to induce a stable baseline of ASR. Thereafter, 18 WN stimuli including the CS or 18 WN stimuli alone were delivered at the intensity of 100 dB. FPS was defined as the difference between the startle amplitude with the presence of the CS and without the presence of the CS.

2.5. Western blot analysis

Rats were sacrificed immediately after the behavioral procedures. The prefrontal cortex

(PFC), hippocampus, striatum, and amygdala were isolated on ice and then stored at -80°C for western blot analysis. The tissue was homogenized using ultrasound in lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM NaF, 1 mM EDTA, 1 mM PMSF, 15 g/ml leupeptin, and 1 mM sodium orthovanadate. The homogenates were then centrifuged for 15 min at 12,500 rpm according to previously described methods [41]. The supernatants were collected, and total protein concentration was determined using the BCA Protein Assay Kit (Beyotime Institute of Biotechnology, China) with the Model MK3 Microplate Reader (Thermo Scientific, USA). Bovine serum albumin was used to prepare a standard curve. The samples were prepared by adding sample buffer and heated for 5 min in boiling water for denaturation. The denatured protein samples were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with the Universal-Mini PROTEAN Tetra System (Bio-Rad, USA). Proteins were electrotransferred onto PDVF membranes (0.2 μm) for 110 min at 250 mA. The membranes were blocked with 5% nonfat milk. The membranes were incubated in the following antibodies: rabbit polyclonal anti-BDNF (Santa Cruz; 1:200 dilution); rabbit monoclonal anti-phospho-CREB (Ser133) (Cell Signaling Technology; 1:400 dilution); rabbit monoclonal anti-CREB (Cell Signaling Technology; 1:400 dilution); and mouse monoclonal anti- α -tubulin (Sigma-Aldrich; 1:10,000 dilution). After the membranes were incubated with an HRP-conjugated secondary antibody (Sigma-Aldrich), the immunoreactive bands were detected using an ECL Kit (Millipore). The intensity of the immunoreactive bands was quantified using densitometry and Quantity One software. The relative band intensity of the BDNF signal was quantified relative to the intensity of α -tubulin.

2.6. Statistical analysis

Data were presented as mean $\pm$ SEM. Quantitative data were statistically analyzed using a one- or two-way ANOVA followed by LSD post-hoc comparison between groups. $P < 0.05$ was considered as a significance.

3. Results

3.1. Inhibition of PDE10A reverses the effect of REM-SD on long-term fear memory

We first investigated the effect of REM-SD on long-term fear memory as measured using FPS (fear-potentiated startle). REM-SD was conducted immediately after the fear conditioning training. The experimental procedure is illustrated in Fig. 1A. To ensure each group of rats had a similar overall mean startle amplitude and shock reactivity, startle response curves (Fig. 1B) and shock reactivity (Fig. 1C) were measured. Two-way ANOVA analysis revealed that there was a significant difference among the groups based on the different sound intensities ($F=144.8$, $P < 0.0001$), but there was no difference among the groups prior to the treatment ($F=1.785$, $P=0.1184$), and there was no sound intensity $\times$ treatment interaction ($F=1.182$, $P=0.2902$). For shock reactivity, there was no significant difference between the treatments ($F=0.5040$, $P=0.7715$, one-way ANOVA) across the training days. There was no difference in the behavioral response to the sound startle stimuli and foot shock among the groups (Fig. 1B). MP-10 treatment did not affect the mean startle amplitude in the absence (tone alone) or presence (CS+tone) of the CS (Fig. 1D). The FPS test measures the difference in the presence

and absence of the CS, which is used as an index of fear memory. We found that REM-SD decreased the mean startle amplitude, but MP-10 treatment significantly increased the mean startle amplitude, which was tested using the FPS test ($F=7.047$, $P<0.001$, one-way ANOVA) (Fig. 1D), which suggests that MP-10 attenuates the REM-SD-induced impairment in long-term fear memory. Therefore, our data suggests that the decreased magnitude of the FPS is mainly due to impairment in learning and memory induced by sleep deprivation but not the auditory sense or an inability to sense the foot shock.

3.2. The effect of REM-SD and MP-10 on the expression of pCREB and CREB

To identify the effect of REM-SD on synaptic plasticity, we examined the expression of cyclic AMP responsive element binding protein (CREB) and its phosphorylated counterpart (pCREB). REM-SD did not significantly alter the total CREB protein levels in the PFC (Fig. 2A), hippocampus (Fig. 2B), striatum (Fig. 2C) and amygdala (Fig. 2D). However, the levels of pCREB were decreased in the hippocampus (Fig. 2B) ($*P<0.05$, $**P<0.01$) and striatum (Fig. 2C) ($*P<0.05$, $**P<0.01$) but not in the amygdala (Fig. 2D) in the REM-SD rats compared with the CON and LP rats. However, 1 or 2 mg/kg of MP-10 reversed the decrease of pCREB in the hippocampus ($*P<0.05$, $**P<0.05$) and striatum ($*P<0.05$, $**P<0.01$) induced by REM-SD.

3.3. The effect of REM-SD and MP-10 on the expression of BDNF

BDNF is an important target gene of CREB and plays an essential role in the persistence of long-term memory. We found that REM-SD had no effect on BDNF in the PFC (Fig. 3A), but induced a decrease in BDNF in the hippocampus (Fig. 3B) ($*P<0.05$, $**P<0.01$), striatum (Fig. 3C) ($*P<0.05$, $**P<0.01$) and amygdala (Fig. 3D) ($**P<0.01$, $***P<0.001$) compared with the CON and LP rats. MP-10 reversed the REM-SD-induced decreases in BDNF in hippocampus ($*P<0.05$, $**P<0.01$), striatum ($***P<0.001$) and amygdala ($**P<0.01$, $***P<0.001$). Although there was an increase in BDNF in the PFC of the REM-SD+MP-10 group compared with the REM-SD group ($P=0.4036$, $F=1.113$), this result was not statistically significant.

4. Discussion

In this study, we found that 6 h of REM sleep deprivation caused deficits in long-term fear memory in rats. This deficit was accompanied by a decrease in pCREB in the hippocampus and striatum, and a decrease in BDNF in the hippocampus, striatum and amygdala. Inhibition of PDE10A by MP-10 prevented the deficit in fear memory induced by REM-SD, which was accompanied by an increase in pCREB and BDNF in some brain areas in a dose-dependent manner. Our findings suggest that REM-SD alters the consolidation of long-term fear memories and that MP-10 inhibits long-term fear memory potentially through increased expression of pCREB or BDNF in the hippocampus, striatum and amygdala.

Most studies have assessed fear memory by measuring freezing. Although this paradigm is commonly used to measure learned fear in rats, it is difficult to accurately monitor fear memory in hypoactive or hyperactive rats in the current experimental paradigm. Therefore, we measured fear potentiation using the acoustic startle response (FPS), which measures an involuntary reflex and does not depend on assessing freezing behavior. In our observations, a

short period of REM-SD (6 h) immediately after fear-conditioning training resulted in a deficit in long-term memory. Interestingly, application of a PDE10A inhibitor MP-10 dose-dependently restored the REM-SD-induced impairment in long-term memory.

PDE10A is highly expressed in the striatal complex [24, 25] and catalyzes cAMP and cGMP [26-29]. Inhibition of PDE10A leads to higher levels of cAMP than cGMP, and PDE10 inhibitors have shown potential for the treatment of cognitive dysfunction [30, 32, 33]. In addition, PDE10 inhibition can reverse the memory deficits induced by scopolamine and MK-801 [34]. Our study further provides evidence that inhibition of PDE10 can reverse REM-SD-induced memory deficits.

It is well known that synaptic structural changes are the neural basis for long-term learning and memory [14]. CREB, which is a major transcription factor, plays a crucial role in the formation of synapses and regulation of synaptic plasticity in the consolidation of fear memories [14, 17, 18, 42-44]. *BDNF*, which is a CREB target gene, is also critical for synaptic plasticity and memory processing in the adult brain [45-47]. SD decreases the levels of pCREB and BDNF [20], and affects the high frequency stimulation-induced increases in pCREB and BDNF levels in the hippocampus [48]. We further observed that REM-SD could impair long-term fear memory accompanied by decreased levels of pCREB and BDNF in hippocampus. We further observed that MP-10 could reverse the deficits in fear memory potentially by increasing the levels of pCREB and BDNF in hippocampus, which suggests a role of PDE10 in fear memory.

It is well documented that the amygdala plays an essential role in emotional learning and memory [49-52]. The neurons in the amygdala exhibit associative plasticity during aversive conditioning [53, 54]. It was reported that there is no significant alteration in pCREB in the basolateral amygdala complex after 5 h of total SD [20], which is consistent with our data. In contrast, it was reported that SD reduces pCREB expression in the central and basal nucleus in a fear-conditioning task [55]. The discrepancy between these findings may be due to the (1) different length of SD (the duration of REM-SD is 72 h but only 6 h in the previous study) or (2) the different methods of fear memory training (contextual fear conditioning task, which is more hippocampus-dependent, compared with FPS). Our findings suggest that there was no change in the expression of pCREB/CREB, but there was an increase in the expression of BDNF in the REM-SD/MP-10 group in the amygdala. We suggest that the increase in BDNF levels is a result of the fear conditioning training and not the MP-10 treatment because PDE10A immunoreactivity and mRNA was scarce in the amygdala. [25, 56, 57]. In addition, that fact that we observed no changes in pCREB in the amygdala and PFC may be due to the low abundance of PDE10A in the amygdala [24], which explains the low response to MP-10. Because fear memory is highly associated with amygdala and hippocampus, our observation that BDNF did not change in the PFC may explain the minimal effect of fear memory on the PFC. It was reported that fear conditioning training upregulates BDNF mRNA in the amygdala [58], which further suggests that the REM-SD-induced decrease in BDNF can be increased by fear-conditioning training.

In addition, we analyzed the expression of pCREB/CREB and BDNF in the dorsal striatum. The dorsal striatum is a structure of the basal ganglia and is involved in various forms of learning and memory [59]. We found that there was a decrease in pCREB/CREB and BDNF expression with 6 h of REM-SD. MP-10 reversed the deficit in fear memory by increasing the

ratio of pCREB/CREB and BDNF in the striatum. It was reported that post-training lesions of the central nucleus of the amygdala could completely block both contextual freezing and FPS [60]. Post-training lesions of the dorsal hippocampus attenuated contextual freezing, but had no effect on FPS, which suggests that FPS is not affected by damage to the hippocampus alone. Taken together with our data, we propose that the hippocampus, striatum and amygdala are also involved in FPS. Further evidence of this involvement was obtained by utilizing MP-10, a PDE10A-specific inhibitor. MP-10 blocks the metabolism of cAMP and cGMP, particularly cAMP. In turn, cAMP activates protein kinase A (PKA), which leads to phosphorylation of multiple proteins involved in nuclear signaling and fear memory consolidation (such as CREB). The phosphorylation of CREB enhances the transcription of its target genes [61], such as *c-fos*, growth factors, enzymes, and neurotransmitters [62, 63]. In our observations, MP-10 reverses the deficits in long-term fear memory that were accompanied by elevated levels of pCREB/CREB and BDNF in the striatum, hippocampus and amygdala, which further supports that in addition to the hippocampus, the striatum and amygdala are also involved in fear memory as measured by FPS.

5. Conclusion

In this study, we found that REM sleep deprivation is associated with deficits in long-term fear memory as measured with the FPS test. This deficit is accompanied by a reduced ratio of pCREB/CREB and BDNF in the hippocampus, striatum and amygdala. MP-10 reversed this deficit by elevating the levels of pCREB and BDNF in these brain areas. Therefore, PDE-10 and its regulation of pCREB and BDNF play a role in SD-induced deficits in long-term fear memory.

Conflict of interests

We declare that we have no conflict of interest.

Acknowledgments

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Figure legends

Fig. 1. REM-SD caused deficits in long-term fear memory, and MP-10 treatment prevented these deficits as measured with the FPS test. (A) The timeline of behavioral experiments. (B) The startle response curves for all six groups of rats, which illustrate that the overall mean startle amplitudes were similar. (C) Behavioral responses to foot shock showing that there were no differences in the groups. (D) Histograms of the mean startle amplitude of the rats in the absence (tone alone) or presence (CS+tone) of the CS. Fear-potentiated startle was measured in the presence and absence of the CS. Data are presented as the mean $\pm$ SEM (N=8~9). ** $P < 0.01$, *** $P < 0.001$ compared with the REM-SD group, one-way ANOVA followed by LSD post hoc test. The startle response curves and shock reactivity are depicted in arbitrary units. MP-10 was injected at 0.5, 1 and 2 mg/kg intraperitoneally. CON: control; LP: large platform.

Fig. 2. The effects of REM-SD and MP-10 on the levels of pCREB in fear memory-related brain regions. MP-10 was injected at 0.5, 1 and 2 mg/kg intraperitoneally 30 min before fear-conditioning training. The rats were then sacrificed immediately after the FPS test. The ratio of pCREB to CREB in the animals subjected to REM-SD with or without MP-10 treatment is shown. (A) The prefrontal cortex, (B) hippocampus, (C) striatum, and (D) amygdala. Data are presented as the mean $\pm$ SEM (N=3). * $P < 0.05$ vs. control or REM-SD group and ** $P < 0.01$ vs. control or REM-SD group using a one-way ANOVA followed by LSD post hoc test.

Fig. 3. The effects of REM-SD and MP-10 on the levels of BDNF in fear memory-related brain regions. REM-SD did not decrease BDNF level in the PFC (A), but caused decreases of BDNF in the hippocampus (B), striatum (C) and amygdala (D) compared with CON or LP rats. MP-10 reversed the REM-SD-induced decrease in BDNF. Data are presented as the mean $\pm$ SEM (N=3). * $P < 0.05$ vs. control or REM-SD group; ** $P < 0.01$ vs. control or REM-SD group; *** $P < 0.001$ vs. control or REM-SD group using a one-way ANOVA followed by LSD post hoc test.

Figure 1

Figure 2

Figure 3