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ORIGINAL ARTICLE

# Protective effect of melatonin against methamphetamine-induced attention deficits through miR-181/SIRT1 axis in the prefrontal cortex

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## Abstract

**Introduction** Methamphetamine (METH) is an addictive psychostimulant with deleterious effects on the central nervous system. Chronic use of METH in high doses impairs cognition, attention and executive functions, but the underlying mechanisms are still unclear. Sirtuin 1 (SIRT1) is a post-translational regulator that is downregulated following METH neurotoxicity. Melatonin is a neuroprotective hormone that enhances mitochondrial metabolism. Here, we evaluated the effect of melatonin on METH-induced attention deficits disorder and the involvement of the miR-181/SIRT1 axis in melatonin neuroprotection.

**Methods and results** METH at a dose of 5 mg/kg was injected for 21 consecutive days. The animals were assigned to receive either melatonin or the vehicle after METH injections. Attention levels were evaluated with abject-based attention test. In the prefrontal cortex, the expression levels of miR-181a-5p, SIRT1, p53 and CCAR2, as well as the mtDNA copy numbers were evaluated using qRT-PCR and western blotting. The outcomes revealed that melatonin treatment following METH injections improved METH-induced attention deficits. METH toxicity can be associated with changes in the miR-181/SIRT1 axis, elevated levels of p53 and COXII, and decreased levels of mtDNA in the prefrontal cortex of adult rats. Interestingly, administration of melatonin can improve the expression of these molecules and reduces the toxic effects of METH.

**Conclusion** Melatonin ameliorated the neurotoxicity of METH in the prefrontal cortex and the miR-181/SIRT1 axis is involve in the protective effects of melatonin. However, melatonin can be potentially administrated to improve attention impairment in METH use disorders.

**Keywords** Melatonin · Attention · Methamphetamine · Prefrontal cortex · Sirtuin 1

## Introduction

Methamphetamine (METH) is a potent addictive psychostimulant whose addiction is on the rise and has become a major public health issue[1; 2]. METH easily passes the blood-brain barrier and alters the function of monoaminergic synapses, which causes euphoria, excitement, and energy uplift. METH increases oxidative stress, metabolic alterations and inflammation, leading to long term damage to the nervous system [3]. Studies have shown that chronic METH use results in a lack of attention and impaired concentration. These effects can persist even after METH abuse, making it a significant concern for long-term METH addiction [2]. Chronic use of METH reduces dopamine and serotonin neurotransmission and reduces neuronal survival in the prefrontal cortex (PFC) which result in suppression of PFC activation and causes significant atrophy in this area of brain [4, 5].

Interestingly, there are evidences that mitochondrial function and biogenesis represent both direct and indirect targets of METH neurotoxicity [1]. It has also been shown that exposure to METH leads to decrease in mitochondrial biogenesis and mitochondrial dysfunction, which prevents metabolic homeostasis and eventually increases oxidative

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stress [1]. METH-mediated ROS production leads to mitochondrial degeneration followed by the induction of apoptosis in neurons [6]. These documents provide a potential molecular mechanism for METH-related neural and cognitive impairment as well as insights into therapeutic strategies against the METH neurotoxicity.

Sirtuin 1 (SIRT1), a highly conserved $NAD^+$ dependent deacetylase, is a post-translational mediator that regulates the function of various transcription factors [7]. SIRT1 and its substrates can be translocated to the mitochondria. Within mitochondria, SIRT1 and its downstream proteins are associated with mitochondrial DNA (mtDNA). The expression of SIRT 1 is necessary to increase the number of mtDNA copies by orchestrating the molecular pathways involved in mtDNA replication. Additionally, SIRT1 modulates cellular metabolism, oxidative stress and inflammatory mechanisms, all of which are intricately linked to mitochondrial function [8]. In the central nervous system (CNS), the activity of SIRT 1 directly links the cellular metabolic situation to the regulation of gene expression to protect against neurotoxicity [9]. SIRT1 deacetylation of p53 inhibits its activation capacity and promotes cell survival. Moreover, SIRT1 activity inhibits mitochondria-mediated apoptosis by inhibiting the mitochondrial translocation of p53 [10]. Cell cycle and apoptosis regulator 2 (CCAR2) suppresses the deacetylase function of SIRT1 and repression of p53 and promotes p53-mediated apoptosis under stress conditions in neurons. Researches have suggested that CCAR2 promotes apoptosis and reduces neuronal survival [10].

It has been reported that METH reduces SIRT1 levels which is associated with mitochondrial damage and oxidative stress [11]. Moreover, chronic use of METH increased oxidative stress and p53 expression and subsequently enhanced apoptosis in neurons [12]. Deletion of p53 in neurons has been shown to prevent synaptic damages in METH-treated animals [13]. In addition, the administration of METH increased the expression of p53 in the PFC, which has been associated with attention disorders in mice [3].

Melatonin (N-acetyl-5-methoxytryptamine), an amphiphilic hormone naturally produced by the pineal gland, has gained attention owing to its potential neuroprotective properties. Melatonin is easily distributed in the CNS and protect neurons against metabolic damages [14]. Melatonin has been found to enhance SIRT1 expression that promote neural survival against neurodegeneration [15]. It has been reported that melatonin increases SIRT1 expression and mtDNA copy number in the hippocampus, improved memory and anxiety, and diminished hippocampal cell damage in animal models [16]. Moreover, melatonin improves working memory impairment and spatial learning and memory impairment by reducing neuroapoptosis, and neuroinflammation through the SIRT1 signaling pathway [15]. Melatonin suppresses neurodegenerative pathways and induces neuronal repair and mitochondrial function via suppression of p53 expression [17]. Although there is no study on the effects of melatonin on CCAR2 expression levels, it is reported that CCAR2 deficiency alters the effects of melatonin on apoptosis in cancerous cells which proposed the involvement of CCAR2 in melatonin effects [18]. However, further studies are required to figure out the precious mechanisms of this pathway.

MicroRNA-181 level is crucial in regulation of neuroprotective pathways and cognitive functions. This microRNA inhibits the expression of proteins involved in neuronal survival and mitochondrial biogenesis and causes increased oxidative stress in neurons [19]. Studies revealed that miR-181 acts as a negative regulator of SIRT1 expression and indirectly alters p53 expression. This microRNA contributes in various neuronal damage pathways through targeting SIRT1 [16]. Increased miR-181 level, is associate with synaptic dysregulation and metabolic damages. miR-181 down-regulation has been seen to reduce oxidative stress, neuronal death and neuroinflammation [20].

We aimed to investigate a direct relationship between miR181, SIRT1, CCAR2, and p53 and mtDNA amount in attention deficits induced by methamphetamine neurotoxicity. Additionally, we explored the potential protective effects of melatonin against methamphetamine induced neurotoxicity and its impact on the expression of SIRT1, miR-181, CCAR2, and p53 as well as mitochondrial biogenesis.

## Materials and methods

### Animals and experimental groups

All experimental procedures in the present research were performed according to the guidelines for animal experiments approved by the ethics and research committee of Semnan University of Medical Sciences and the guide for care and use of laboratory animals introduced by the National Institute of Health. In the current study, 32 adult male Wistar rats (Pasteur Institute, Tehran, Iran) weighing 200–220 g were randomly placed in transparent cages with free access to food and tap water. The cages were maintained at $22 \pm 2$ °C with a light/dark cycle of 12:12 h (lights on - at 07:00). One week before the start of the procedure, the cages were transferred to the laboratory environment for habituation and maintenance.

The rats were assigned randomly in four groups with eight animals in every group; The "control" group that received vehicles of administrated agents (saline and DMSO1%) for 21 days. The control group was design to ensure that the observed effects are indeed due to the administration of

melatonin and METH and not confounded by other variables. “Melatonin group” that received saline followed by melatonin. “METH group” that received methamphetamine daily for 21 days) “METH + melatonin group” that received methamphetamine and melatonin for 21 consecutive days. From each group, the brain tissue of four animals was randomly prepared for western analysis and four animals for real-time PCR analysis. The duration of 21 consecutive days for administration of METH was chosen to establish a rat model of methamphetamine chronic abuse and associated brain damage induced by METH. In order to eliminate the problem of bias, the researchers who conducted and analyzed the behavioral tests and the molecular studies were completely blind about the animal groups. The outcomes of melatonin injection alone and METH group, were compared with the control animals. The control group was under the same conditions and under the same treatment as other groups. The only difference was that they received METH vehicle (saline) and melatonin vehicle (DMSO 1%) to ensure that the effects of the prescribed drugs did not interfere with environmental factors and drug vehicles.

## Chemicals

Dimethyl sulfoxide (DMSO) and melatonin were obtained from Santa Cruz Biotechnology (California, USA). Methamphetamine was injected intraperitoneally every morning between 9:00 and 10:00 a.m. To prevent interference in the circadian rhythm, melatonin was injected intraperitoneally every afternoon from 5:00 pm to 6:00 pm for 21 consecutive days. The final administrated melatonin dose was 10 mg/kg. In previous study, it has been reported that melatonin at a dose of 5 mg/kg is not effective against METH neurotoxicity effects [21]. In addition, it has been seen that melatonin at the dose of 10 mg/kg is both effective and safe against the METH induced cognitive impairment [14]. Melatonin was dissolved in 1% DMSO and diluted with saline to a final concentration of 2 mg/ml. Then freshly prepared melatonin (10 mg/kg body weight/day) was administrated. Methamphetamine was dissolved in normal saline and injected at a final dose of 5 mg/kg. The neurotoxic effects of this dose of methamphetamine have been reported previously [22].

## Behavioral test

Attention was assessed using an object-based attention test (Fig. 1a). For this purpose, the previously described protocol was applied [23]. This test was conducted based on experience with a rectangular apparatus with two chambers: an exploration chamber (60 × 40 × 50 cm) and an experimental chamber (30 × 40 × 50 cm) [23].The video recording camera was located above the instrument. The rats were familiarized with the environment of the rooms the day before the procedure. During the habituation phase, the rats explored both empty rooms during a 10-minute familiarization session. On the test day, during the acquisition phase, rats were given a 5-minute session in the exploration room to explore five objects with different shapes and similar colors (1, 2, 3, 4, and 5).Animals spend equal amounts of time on each object in this phase [24]. The time that animal spends on each object in the exploration phase is recorded as the exploration time which is obtained via dividing the total time of exploration next to the objects by the number of objects. Immediately after the acquisition phase (< 10 s) the retention phase was began and one of the objects used in the acquisition phase (object number 5) was selected and entered into a position parallel to the previous position in the test room. In addition, a sixth with a new shape and the same color as the previous objects was placed in the test room (object 6). The rat was immediately transferred to the test room to start the retention phase. During the retention phase, both objects were examined by rat for 5 min. The recognition index in the retention phase was analyzed as the ratio (T6 × 100) / (T5 + T6), where T5 and T6 are the time spent on objects 5 and 6, respectively.

## PFC tissue collection

Following attention test, rats were anesthetized with $CO_2$ and their brains were isolated and immediately maintained in the cold phosphate-buffered saline. PFC tissues were separated and frozen quickly in liquid nitrogen compartment and then stored at − 80 °C.

## Gene expression analysis

The total RNA extraction Mini Kit (YTA, Iran) was used for total RNA extraction. RNA concentration and quality were examined using a NanoDrop spectrophotometer (Thermo. Scientific, USA). For quantitative PCR for microRNA, we used a specific stem-loop RT primer under conditions of 37 °C for 60 min and then 85 °C for 5 min. The cDNA related to miR-181a-5p was then used for quantitative PCR under the following conditions: 95 °C for 15 min and 45 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. For mRNA expression analysis, 200 ng of extracted RNA was reverse transcribed by cDNA synthesis kit (YTA, IRAN) with the following condition: 37 °C for 60 min, 85 °C for 5 s, and 4 °C for 5 min. For future molecular experiments, the obtained cDNAs were kept at -20 °C. qRT-PCR was performed with RealQ Plus 2x Master Mix Green (Ampliqon, Denmark) in a LightCycler® 96 System Detector (Roche, Switzerland) using the following program: 95 °C for 5 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s and

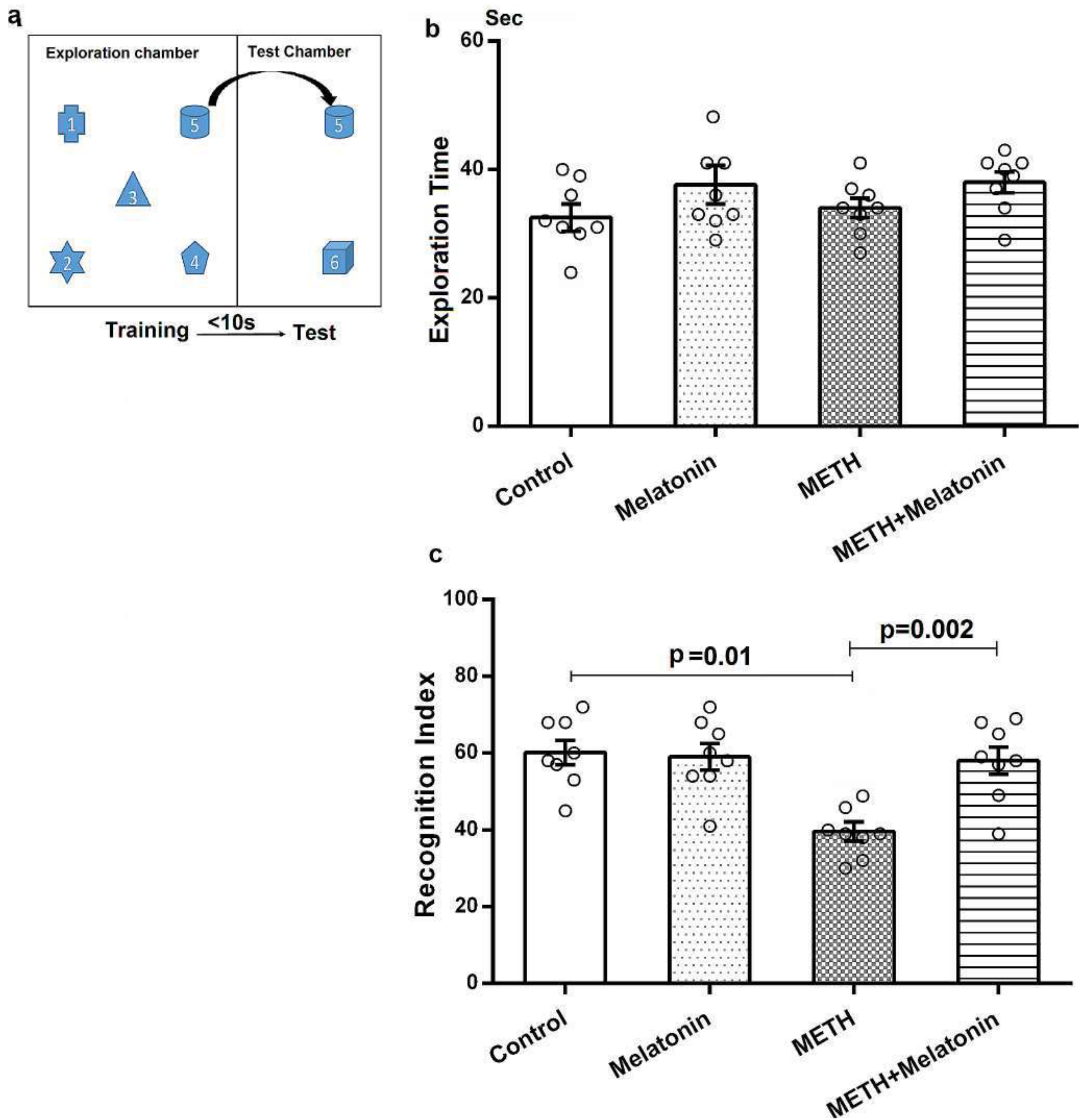


**Fig. 1** Evaluation of attention using object-based attention test. (**a**) Design and procedures of object-based attention test. (**b**) The time spent by the animal to search for each object in the trial phase is shown as exploration time. (**c**) Measuring the recognition index in the object-based attention test that shows the level of the animal's attention. Values are presented as mean ± SEM. Significant difference was set as p-value < 0.05

72 °C for 30 s. U6 snRNA was used as the internal control for the expression analysis of the miRNA, whereas GAPDH gene expression was applied as a control for the expression evaluation of the coding RNAs. The relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ analysis method. The primers are listed in the Table 1.

## Quantification of mitochondrial DNA

The mitochondrial DNA was extracted from PFC tissues using the QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. The copy numbers of mtDNA were evaluated by qRT-PCR using SYBR

**Table 1** List of primer sequences used for Real-Time PCR analysis

| Gene | Primer | Sequence |
|---|---|---|
| p53 | F | 5’-AGTCTGTTATGTGCACGTACTC-3’ |
| p53 | R | 5’-TCGTCCAGATACTCAGCATACG-3’ |
| CCAR2 | F | 5’-TGCTACCCAAAGTGTTGAAGC-3’ |
| CCAR2 | R | 5’-GACCTTGAAGGTGCAGTTTGC-3’ |
| SIRT1 | F | 5’-ATACCTTGGAGCAGGTTGCAG-3’ |
| SIRT1 | R | 5’-TGTCATACTTCATGGCTCTATG-3’ |
| GAPDH | F | 5’-TGTGACAAAGTGGACATTGTTG-3’ |
| GAPDH | R | 5’-TCCTGGAAGATGGTGATGGGT-3’ |
| COXII | F | 5’-AGGACAGCATTCCAAATGTCC-3’ |
| COXII | R | 5’-CAAAGTAGTCATGCAAGCTGG-3’ |
| miR-181a-5p | loop | 5’-GTCGTATCCAGTGCAGGGTCCG AGGTATTCGCACTGGATACGACTTA AGG-3’ |
| miR-181a-5p | F | 5’-GTCGTATCCAGTGCAGGGT-3’ |
| miR-181a-5p | R | 5’-AACAAGAGATGGGCAACCAAG-3’ |

*CCAR2* Cell division cycle and apoptosis regulator 2; *SIRT1* Sirtuin1; *GAPDH* Glyceraldehyde 3-phosphate dehydrogenase; *COXII* Cytochrome c oxidase subunit 2

Green detection method. Primers for mitochondrial gene, cytochrome c oxidase II and nuclear gene, GAPDH for mtDNA evaluation are presente in Table 1.

### Western blotting analysis

Tissues were lysed using Tris-HCl, SDS, Triton X-100 buffer in the presence of protease inhibitor (Roche, Penzberg, Germany). For each group, the same quantity of protein was calculated using the Bradford method, loaded in the wells in a 12.5% (SDS)-PAGE gel and then transferred to a PVDF membrane (Merck Millipore, USA). Attempts were made to minimize the loading errors. To prevent misplaced joints, covering with 2% skim milk was used. Then the samples were incubated with primary SIRT1 and β-actin antibodies (Cell Signaling Technology, USA). The membrane incubated with anti-rabbit IgG horseradish peroxidase conjugated antibody (Cell Signaling Technology, USA). ECL reagents (Pars Co, Iran) were used to detect immunoreactive polypeptides. The bands were visualized and fixed Using X-ray films. The SIRT1 band in each group was normalized to the respective β-actin bands. Blot intensity was analyzed by ImageJ software.

### Statistical analysis

All data were processed using SPSS software and are shown as mean ± standard error of the mean (SEM). The diagrams were designed using GraphPad Prism software. One-way analysis test of variance (ANOVA) and Tukey’s post-hoc test were performed to compare groups. Statistical significance was set at p-value $< 0.05$.

## Result

### The effect of melatonin on attention deficits induced by METH toxicity

Animals in all groups explored defined objects in the same quantity of time during the acquisition phase (Fig. 1b). There was no significant difference between groups in exploration time ($F(3,28) = 1.58$, $p = 0.21$). In the retention session, rats in METH group still spent more time with the previous object compared to the new object, while control and METH + Melatonin groups spent fewer time next to the previous object. The result demonstrated that animals in the METH group explored the objects with unfocused attention during the training session, which leads to an incapability to discriminate the previously explored object in the test session. One-way ANOVA showed that there was a significant difference between groups in recognition index ($F\ (3,28) = 9.29$, $p < 0.001$). Consequently, METH causes decrease in the exploration index in the retention phase of the object-based attention test ($39.62 \pm 3.05$ vs. $60.12 \pm 3.12$, $p = 0.001$, Fig. 1c). Melatonin administration after METH injection prevented the deleterious effect of METH on exploration index ($58.00 \pm 3.57$ vs. $39.62 \pm 3.05$, $p = 0.002$, (Fig. 1c). Result of object-based attention test revealed that melatonin is protective against deleterious effect of METH on attention.

### Melatonin effects on miR-181/ SIRT1 axis in the PFC following METH toxicity

It has been demonstrated that miR-181 directly targets SIRT1 expression[25; 26]. Using one-way ANOVA analysis we found significant differences between the groups in miR-181 expression levels ($F(3,12) = 10.7$, $p = 0.001$). METH injection (5 mg/kg for 21 days) increased miR-181 expression levels in the PFC in comparison with the control group ($2.33 \pm 0.31$ vs. $1.01 \pm 1.1$, $p = 0.003$, Fig. 2a). Melatonin administration prevented the enhancement of miR-181 levels in the PFC of melatonin + METH group compared to METH group ($2.33 \pm 0.31$ vs. $1.33 \pm 0.17$, $p = 0.021$). According to these results, administration of melatonin following METH injection diminished the level of miR-181 (0.57 fold) in the PFC.

Data showed that the SIRT1 expression levels were significantly different between the groups ($F(3,12) = 23.41$, $p < 0.001$). The injection of METH decreased SIRT1 levels in the PFC compared to that in control group ($0.30 \pm 0.02$ vs. $1.01 \pm 1.1$, $p < 0.001$, Fig. 2b). Melatonin administration inhibited the decrease in SIRT1 in the PFC of melatonin + METH group compared to METH group ($0.96 \pm 0.6$ vs. $0.30 \pm 0.02$, $p < 0.001$). The results showed that melatonin

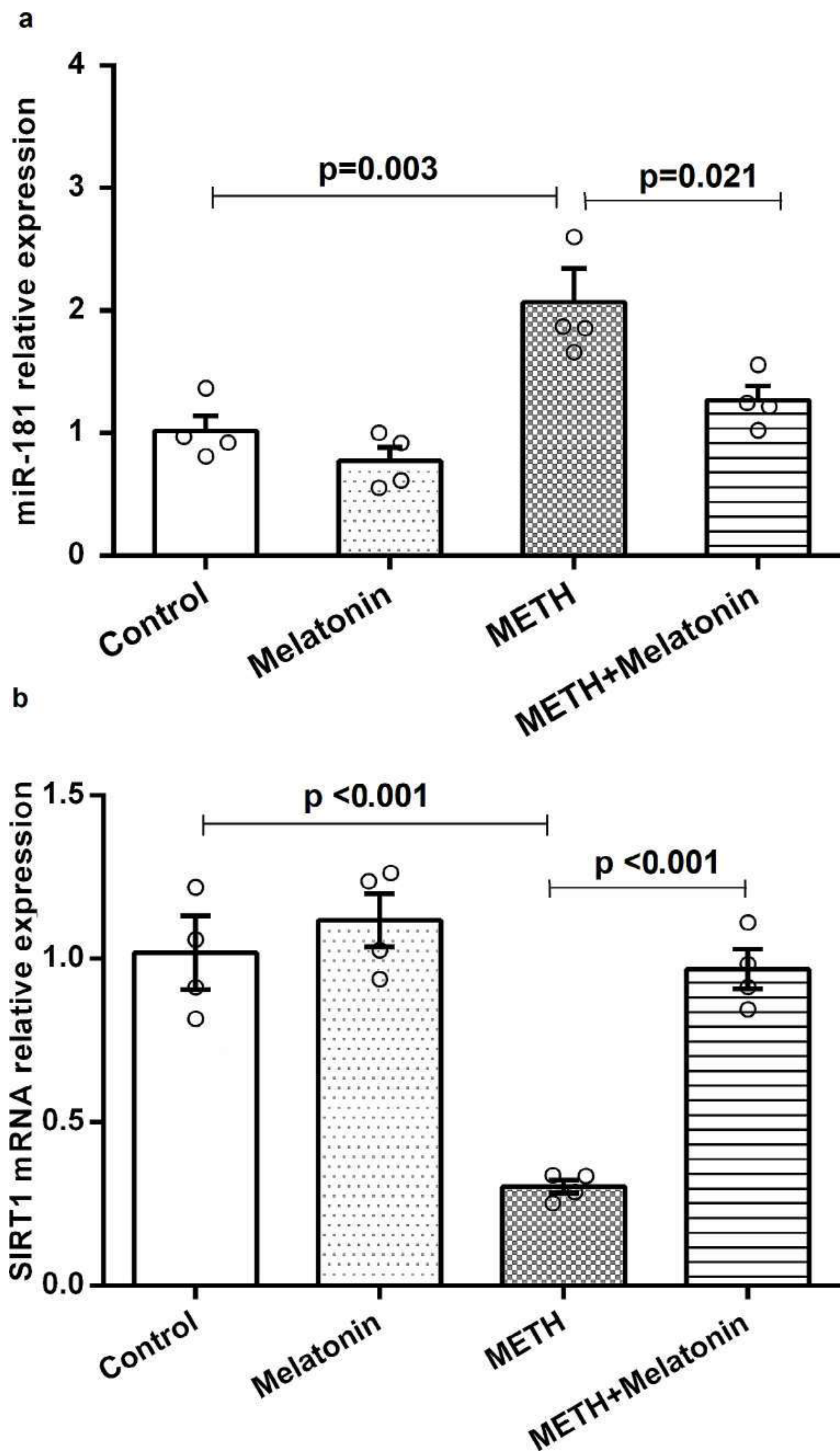


**Fig. 2** Effect of melatonin on miR181/SIRT1 axis in PFC. (**a**) Evaluation of miR-181 levels using qRT-PCR. (b) Evaluation of mRNA related to SIRT1 using qRT-PCR. Values are presented as mean ± SEM. Significant difference was set as p-value < 0.05

administration after METH injection, increased the level of SIRT1 (3.2 fold) in the PFC.

There was a significant difference between groups relate to data of SIRT1 western blotting ($F(3,12) = 58.62$, $p < 0.001$). Western blot analysis of SIRT1 protein levels showed that METH reduced SIRT1 protein levels in the PFC compared to the control group ($0.37 \pm 0.03$ vs. $0.88 \pm 0.02$, $p < 0.001$, Fig. 3). Melatonin prevented SIRT1 reduction in the PFC of melatonin + METH group in comparison with METH group ($0.80 \pm 0.02$ vs. $0.37 \pm 0.03$, $p < 0.001$). These data showed that melatonin administration following METH injection increased the level of SIRT1 protein (2.1 fold) in the PFC.

### The effect of melatonin on p53 and CCAR2 expression levels in the PFC

p53 is a substrate of SIRT1 that induces apoptosis by direct transcriptional activation of apoptotic proteins [27]. One-way ANOVA analysis showed that p53 levels varied significantly between groups ($F(3,12) = 23.21$, $p < 0.001$). METH (5 mg/kg for 21 days) enhances p53 levels in the PFC in comparison with the control group ($2.29 \pm 0.15$ vs. $1.07 \pm 0.11$, $p < 0.001$, Fig. 4a). Melatonin administration prevented the rise of p53 in the PFC of melatonin + METH group in comparison to METH group ($1.40 \pm 0.14$ vs. $2.29 \pm 0.15$, $p = 0.005$). It can be concluded that administration of melatonin following METH injection, decreased the level of p53 (0.48 fold) in the PFC and modulated the damage caused by METH on the PFC.

Moreover, Data analysis showed that CCAR2 levels varied significantly between groups ($F(3,12) = 18.99$, $p < 0.001$). METH enhances CCAR2 levels in the PFC compared to the control group ($1.02 \pm 0.12$ vs. $2.18 \pm 0.17$, $p < 0.001$, Fig. 4b). Melatonin administration modulated the enhancement of CCAR2 in the PFC of melatonin + METH group compared to METH group ($1.40 \pm 0.06$ vs. $2.18 \pm 0.17$, $p = 0.003$). CCAR2 is a crucial negative regulator of SIRT1 [28]. In conclusion, administration of melatonin following METH injection increases the level of CCAR2, which mediates the inhibition of SIRT1 and cellular stress responses.

### The melatonin effect on mtDNA copy number in the PFC following METH toxicity

To examine the mtDNA copy number in the PFC, we evaluated the ratio of mtDNA to nDNA using Real-time PCR as previously described [16]. The mtDNA copy number was significantly different between the groups ($F(3,12) = 21.56$, $p < 0.001$, Fig. 5). METH reduced the mtDNA copy number in the METH group compared to the control group ($0.55 \pm 0.01$ vs. $1.04 \pm 0.06$, $P < 0.001$). Melatonin suppressed the reduction of mtDNA copy number in the METH + Melatonin group in comparison with the METH group ($0.98 \pm 0.04$ vs. $0.55 \pm 0.01$, $P < 0.001$). these data showed that melatonin injection enhanced the mtDNA copy number in the PFC of METH-treated rats.

## Discussion

Attention is a fundamental cognitive process that involves the ability to selectively focus on relevant stimuli while inhibiting distractions. Attention deficit disorder can significantly affect many aspects of daily life, contributing to poor decision-making, reduced productivity, and compromised overall functioning [29]. Attention deficit disorder is a major debilitating factor in addicts, that reduces their ability to socialize and eventually isolates them [30]. In the current study, we reported that METH at a dose of 5 mg/kg for 21 days impaired attention in rats. Obermeit et al. reported that impairments in attention required performances are prevalent among human METH abusers [30]. Moreover, Dean et al. also reported low performance in sustained attention tests in the METH-dependent subjects [31]. Impairment in attention can persist even after expanded periods of abstinence, suggesting long-term consequences of METH effects on cognition [32]. Additionally, METH exposure during the prenatal period is associated with decreased attention during the childhood [33]. These long term consequences of METH deleterious effects on attention imply attention deficit appears to play a crucial role in METH addiction disorder, indicating that targeting attention abilities may help ameliorate sequelae of METH-abuse disorders [32].

In the current study, we observed that METH exposure alters miR-181/SIRT1 axis in rats. Our results in the PFC confirm the study of Rezaeian et al. which also reported that METH decreases the level of SIRT1 in the hippocampus leading to increased anxiety-like behaviors in animal model [34]. Moreover, Wong et al. reported that METH induces cell damage and oxidative stress by reducing SIRT1 levels in lung cells [11]. SIRT1 and its substrate, PGC-1α, mediate the mechanisms involved in mitochondrial metabolism. A relatively interesting and poorly defined feature of SIRT1 function is its presence in the mitochondria. SIRT1 protein is detectable in the mitochondria of different human cell lines as well as in different rat organs. In the mitochondria, SIRT1 associates with mitochondrial DNA (mtDNA) nucleosides and with mitochondrial transcription factor A (TFAM), a vital mitochondrial protein that increases mtDNA copy number to promote mitochondrial biogenesis [8]. Mitochondrial