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Experimental Research Article

Melatonin Attenuates Methamphetamine Neurotoxicity through inhibition of NLRP3 and pyroptosis pathway

Seyed Khalil Rashidi[1], Mitra Ansari Dezfouli[2*], Fariba Khodagholi[3], Mehdi Dadashpour[1], Ali Akbar Shabani[1*]

1. Department of Medical Biotechnology, Faculty of Medicine, Semnan University of Medical Sciences, Semnan, Iran
2. Department of Neurology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
3. Neuroscience Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

ABSTRACT

Introduction: Methamphetamine (Meth) is a highly addictive psychostimulant and induces neuroinflammatory responses. Melatonin is a neurohormone that has protective effects and reduces inflammation in the central nervous system. Our study focused on the melatonin effect on memory impairment, NLRP3/IL-1β axis, and gasdermin D and caspase-1 expression in the hippocampus of a rat model of Meth use.

Methods: Meth and melatonin were administered to the rats for 21 consecutive days. The memory was evaluated using alternation behavior in Y-maze. NLRP3 and IL-1β were assessed by western blotting and ELISA, respectively. Gasdermin D and caspase-1 expression levels were evaluated using qRT-PCR.

Results: The NLRP3 and IL-1β were elevated in the hippocampus following Meth injection. Moreover, Meth increased gasdermin D and caspase-1 expression levels. After 21 days of Meth use, memory impairment was seen in the Y-maze test. Melatonin significantly improved memory and decreased the expression of NLRP3, IL-1β, gasdermin D, and caspase-1 in the hippocampus.

Conclusion: Our study revealed that inflammasome formation and pyroptosis pathway are involved in Meth-induced neurotoxicity. Melatonin may be a potential treatment against neurotoxicity and cognitive disorders caused by Meth.

Keywords:
Methamphetamine
Melatonin
NLRP3
Interlukine-1β
Working memory
Pyroptosis

Introduction

Methamphetamine (Meth), a derivative of amphetamine, is a highly addictive psychostimulant and the second most widely used illegal drug. (Dobšíková et al., 2023; Paknahad et al., 2021). Meth is also prescript for the treatment of diseases such as obesity, narcolepsy, and attention problems(Degenhardt et al., 2010). Despite the potential benefits in the treatment of some diseases, Meth abuse is associated with various adverse effects including learning and memory impairment, anxiety, depression, and psychosis(GLASNER-DWARDS et al., 2010). Neuroimaging and psychological assessments

* Corresponding author: Mitra Ansari Dezfouli, ansaridezfouli-m@ajums.ac.ir
Ali Akbar Shabani, aashaebani@yahoo.com

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have revealed the neurological effects of chronic Meth abuse. Extensive research has emphasized the neuroinflammatory effects of Meth, particularly on dopaminergic and serotonergic systems in the brain (Ares-Santos et al., 2014; Kaushal and R Matsumoto 2011). Meth-induced degeneration of dopaminergic neurons is mediated by oxidative stress and inflammatory signals (Ruan et al., 2020). Although the deleterious effects of Meth on the central nervous system are well documented, few studies have specifically examined neurotoxicity and its effect on neuroinflammation and pyroptosis in the hippocampus, a critical region of the brain for learning and memory (Polvat et al., 2023).

Emerging evidence implicates inflammasome-related complexes, including NLRP3, IL-1β, caspase-1, and gasdermin D (GSDMD) in inflammatory responses and the pathogenesis of neurotoxicity effects induced by Meth abuse (Kurawa et al., 2023; Yi 2020). Considering the central role of the hippocampus in cognitive functions, a comprehensive evaluation of the effects of Meth on this area of the brain is necessary (Ge et al., 2023). Previous research has shown that suppression of neuroinflammation can significantly reduce Meth-induced damage and the risk of early relapse and stress in animal models (González et al., 2014; Snider et al., 2013). This suggests that targeting neuroinflammation could be a promising strategy against Meth-induced neurotoxicity.

Among important inflammatory molecules, NLRP3, IL-1β, caspase-1, and GSDMD are prominent (Yi 2020). NLRP3 can be activated by toll-like receptor (TLR) agonists. This primary signal is critical for the induction of IL-1β transcription and the NLRP3 inflammasome response to secondary signaling, which includes a wide variety of responses to infections and stress-related stimuli (de Zoete et al., 2014). The NLRP3 inflammasome is an important inflammasome produced by glial cells in the brain and its activation leads to the activation of caspase-1(Lahooti et al., 2021). NLRP3 activation leads to GSDMD processing, which causes the formation of pores in the cellular membrane and IL-1β releasing, ultimately leading to pyroptosis (Shi et al., 2017). Considering the significant effect of NLRP3 on neuroinflammation and neurotoxicity, targeting NLRP3 as a potential strategy to alleviate cognitive impairment in animal studies has been investigated(Shi et al., 2017). Clinical studies have also reported overexpression of NLRP3 in the hippocampus following Meth use (Kurawa et al., 2023). Understanding the specific mechanisms by which methamphetamine affects NLRP3 activation and the downstream inflammatory response is crucial for developing targeted therapies for methamphetamine toxicity. Caspase-1 is an important caspase because it plays a central role in the inflammatory response. It is involved in the activation of pro-inflammatory cytokines, such as interleukin-1β (IL-1β) and interleukin-18 (IL-18), which are key mediators of the inflammatory response(Kurawa et al., 2023).

Interestingly, researchers have shown that Meth can induce apoptosis in a wide range of brain structures, such as the hippocampus, which is critical for cognitive function (Bernheim et al., 2016; Mizoguchi and Yamada 2019). Structural and functional changes in the hippocampus are involved in the cognitive deficits associated with METH use disorder (Mizoguchi and Yamada 2019).

Melatonin is a potent compound that effectively reduces oxidative stress and mitochondrial damage (Dezfouli et al., 2019). It has been shown that melatonin is an endogenous antioxidant and indirectly increases the function of key antioxidative enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (Zhao et al., 2018). In addition, melatonin is involved in circadian rhythm (Burgess et al., 2010), sleep pattern (Burgess et al., 2010), mood disorders (De Crescenzo et al., 2017), cognitive function, neuroprotection (Jumnongprakhon et al., 2017), and cancer(Witt-Enderby et al., 2006). Recent studies have demonstrated that melatonin is an excellent inhibitor of cell death (Rui et al., 2021). Melatonin administration can be used to reduce the effects of oxidative stress on the nervous system and subsequently reduce neuroinflammation (Arioz et al., 2021).

The specific involvement of the NLRP3 inflammasome and its downstream caspase-1/GSDMD axis in Meth-induced behavioral impairment has not yet been elucidated. The present study investigated the involvement of NLRP3/caspase-1/GSDMD and IL-1β signaling and memory impairment in the rat model of Meth use along with the therapeutic potential of melatonin in reducing Meth-induced neuroinflammation. This research contributes to the understanding of the complex mechanisms underlying Meth-induced neuroinflammation and cognitive dysfunction and provides insights into potential therapeutic interventions to reduce these

harmful effects.

Material and Methods

Animals and experimental groups

This study was performed according to the guidelines for the use of laboratory animals introduced by NIH and the ethics and research committee of Semnan University of Medical Sciences. 32 adult male Wistar rats (Pasteur Institute, Tehran, Iran), weighing 210–230 g, were placed in eight cages (four rats per cage) with free access to food and water. This study included four experimental groups: control, melatonin, Meth, and Meth + melatonin. Meth and melatonin were administered to the rats for 21 days. The cages were maintained at 22±2°C with a 12:12 light/dark cycle (lights on at 07:00 a.m.). One week before the injection, the cages were transferred to the laboratory for adjustment. The control group received saline (9-10 a.m.) and 1% DMSO (5-6 p.m.). The melatonin group received saline (9-10 a.m.) followed by melatonin (5-6 p.m.). The Meth group received daily Meth (9-10 a.m.) and DMSO 1% (5-6 p.m.) for 21 days, and the Meth + melatonin group received daily METH (9-10 a.m.) and melatonin (5-6 p.m.). In this experiment, the results of Meth were compared to of those the control group, and the results of the Meth + melatonin group were compared to the Meth group.

Chemicals

Methamphetamine hydrochloride was freshly dissolved in 0.9% saline before each injection. Meth was administrated at a final dose of 5 mg/kg subcutaneously every morning between 9:00 and 10:00. To avoid interference with circadian rhythm, melatonin was injected intraperitoneally (i.p.) every afternoon from 17:00 to 18:00 for 21 days. Melatonin was dissolved in 1% DMSO and injected at the dose of 10 mg/kg.

Behavioral tests

Spatial Working Memory

The behavioral test was done between 11.00 a.m. to12.00 p.m. The Y-maze measures working memory by evaluation of spontaneous alternation behavior. The Y-maze test has the advantage of avoiding unnecessary transfer of stress to the animals while providing memory and motor assessment(d'Isa et al., 2021). The rat alternately explored the three arms to explore the new area. Alternation was performed when the animal visited all three arms in clockwise or counterclockwise directions. The Y-maze test was performed on the 21st day after the injection in the different groups. This maze has three symmetrical arms made of acrylic with dimensions of 50 × 10 cm and a height of 20 cm. The rat was allowed to navigate the arms for 8 min. Alternation behavior and the total number of inputs to the arms were measured. Alternation behavior was monitored using a camera sited at the top of the maze. An arm entry was described as an entry when four paws were reentered. Alternation was described as consecutive entrance into three different arms. The alternation behavior was analyzed using the number of alternations divided by the total number of entries minus two, which was expressed as the percentage.

Immunoblotting analysis

The hippocampal tissue lysis was performed in a buffer containing Tris-HCl, SDS, Triton X-100, and protease inhibitors (Sigma-Aldrich). The protein concentration of each group was assessed using the Bradford Method. The extracted proteins were loaded into the wells of a 12.5% gel (SDS)-PAGE and then transferred to a PVDF membrane (Sigma Aldrich, USA). A coating of 2% skim milk was used as a blocking agent to block non-specific attachment of antibodies to the membrane. Membranes were then incubated with NLRP3 and β-actin primary antibodies. Washing was performed using tris-buffered saline (TBS)-Tween 20. The cells were then incubated with a secondary antibody (horseradish peroxidase). ECL reagents (Amersham Bioscience, Piscataway, NJ, USA) were used to detect immunoreactive polypeptides. The visualized bands were analyzed after exposure to X-ray films. NLRP3 bands in each group were normalized to β-actin bands in the same group. The intensity of the stained blocks was checked using the ImageJ software.

Enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) kit for total IL-1β (Carmania Pars Gene Company, Iran) was used for samples extracted from the hippocampal tissue of rats, according to the protocol.

Real-time quantitative PCR

The caspase-1 and GSDMD mRNA expression levels were assessed in all collected hippocampal samples.

TABLE 1: Primer sequences used for PCR

gene	Forward Primer	Reverse Primer
Gsdmd	5'-ATGTGTCAACCTGTCAATCA-3'	5'- ACACGCAGCATACACATG -3'
Casp-1	5'-TTTGTTACAGAA GATTCTAAGGGA-3'	5'-GTCATCTCCAGAGCTGTGAGAT -3'
Gapdh	5'-CGCCCCTTCCGCTGATGCCCCCA-3'	5'-GGGATGATGTTCTGGGCTGCC-3'

Gsdmd, Gasdermin-D; Casp-1, Caspase-1; Gapdh, glyceraldehyde-3-phosphate dehydrogenase.

RNA extraction from hippocampal tissue was conducted utilizing a total RNA extraction kit (ParsTous, Iran) following the guidelines. The quality of the extracted RNA was assessed with a NanoDrop 2000C. Then, 1 μg of whole RNA was reverse transcribed to cDNA using the Easy cDNA Synthesis Kit (ParsTous, Iran). The resulting cDNA was then utilized in quantitative PCR (qPCR) with RealQ Plus 2x Master Mix Green (Ampliqon, Denmark) on a LightCycler® 96 system detector (Roche, Switzerland) with the specified cycling parameters: primary denaturation at 94°C for sec, then 35 cycles of denaturation at 96°C for 5 sec, annealing at 62°C for 30 sec, and extension at 72°C for 30 sec. GAPDH was employed as the internal control. Relative expression levels were assessed by the $2^{-\Delta\Delta Ct}$ analysis. Detailed primer sequence information is listed in Table 1.

Statistical analysis

Data were processed by SPSS 27.0 software and were shown as the mean ± standard error of mean (SEM). Comparisons between groups were made using one-way analysis of variance (ANOVA) and Tukey's post-hoc test. The level of statistical significance was set at $P< 0.05$.

Results

Effect of Methamphetamine and melatonin on spatial working memory

Spontaneous alternation behavior was evaluated using a Y-maze. Data are presented in Figure 1. Our results showed a significant difference in spontaneous alternation behavior between different groups ($F_{(3,28)}$ =111.4, P<0.001). Specifically, the Meth group revealed decreased alternative behavior in comparison with the control (42.25±1.77 vs. 72.00±2.06, P<0.001). However, spatial working memory was improved in the Meth + melatonin group, as shown by a significant rise in spontaneous alternation behavior compared with the Meth group (70.00±1.41 vs. 42.25±1.77, P<0.001). The spontaneous alternation behavior between the melatonin and saline groups was not different (P=0.93). Furthermore, the total arm entries did not alter among all experimental groups ($F_{(3,28)}$ =0.54, P=0.98)., indicating that overall motor activity in the Y-maze was not affected. This suggests that the reduction in periodic behavior observed in the Meth group is not attributable to alterations in the locomotion of the animals but rather reflects a specific impairment in memory function.

The effect of Methamphetamine and melatonin on IL-1β and NLRP3 levels

Inflammasome signaling has a crucial role in the inflammatory processes (Bulté et al., 2023), and our research has shown a compelling link between chronic Meth use and elevated levels of IL-1β and NLRP3. To comprehensively evaluate the effect of Meth treatment on these inflammatory factors, we used ELISA to quantify the expression level of IL-1β, and western blotting to evaluate the level of NLRP3 in different experimental groups. After behavioral testing and removal of hippocampal tissue, hippocampal lysate from rat models was used in ELISA test, and the analysis of the results clearly showed a significant difference between groups ($F_{(3,28)}$=997.8, P<0.001). As it has been shown in Figure 2, IL-1β concentrations in the Meth group were different in comparison with the control group (19.19± 0.20 vs 9.9±0.9, P<0.001). In addition, western blot analysis and subsequent evaluation with ImageJ software showed a significant difference between groups ($F_{(3,28)}$=111.436, P<0.001). As it is shown in Figure 2, there was a rise in NLRP3 level in the Meth group in comparison with control (0.91±0.01 vs 0.63±0.008, P<0.001). These findings showed that IL-1β and NLRP3 are elevated in the inflammatory cascade, in response to Meth injection. These exacerbated inflammatory responses propose the complex interplay between Meth abuse and inflammasome pathways.

To further investigate the anti-inflammatory role of melatonin in the context of chronic Meth use, we assessed alterations in the levels of IL-1β and NLRP3. Our

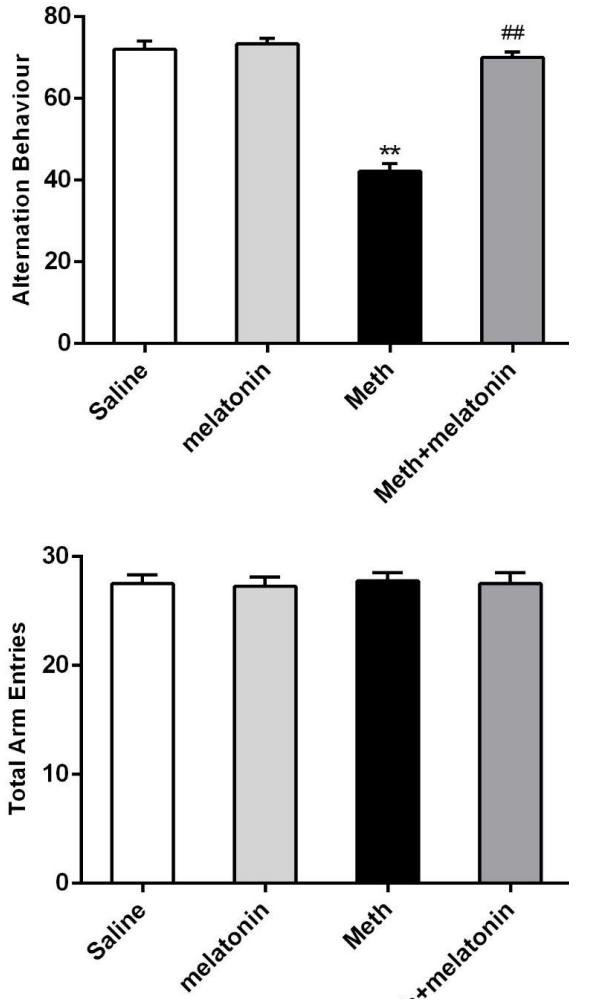

FIGURE 1. Chronic Meth administration significantly reduced alternation behavior in Y-maze. Moreover, melatonin administration improved the alternation behavior in the Meth+melatonin group. **, *P*< 0.001 in comparison with the control; ##, *P*< 0.001 in comparison with the Meth group.

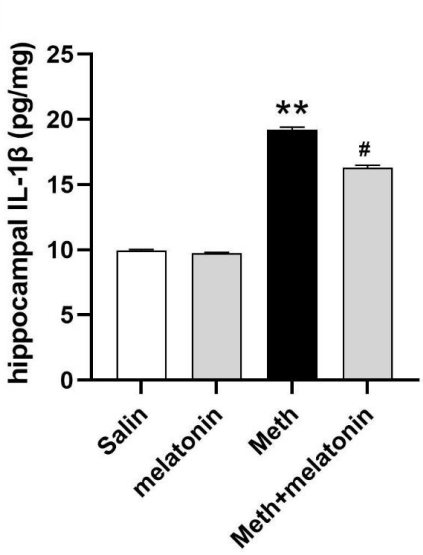

FIGURE 2. IL-1β significantly increased in the Meth group in comparison with the control. Moreover, IL-1β expression decreased in the Meth+melatonin group compared to the group receiving Meth. **, P< 0.001 in comparison with the saline group; #, *P*< 0.05 in comparison with the Meth group.

findings showed a decline in the level of IL-1β expression in the group receiving Meth + melatonin compared to the group receiving Meth (16.3±0.17 vs 19.19±0.20, *P*=0.018). In addition, western blot assay showed a decreased level of NLRP3 in the Meth+melatonin group in comparison with the Meth group (0.82±0.01 vs 0.91±0.01, P <0.001). These results provide compelling evidence for the anti-inflammatory role of melatonin in the context of chronic Meth abuse and valuable insights into its potential as a therapeutic agent to reduce the inflammatory effects associated with Meth use.

Chronic use of Methamphetamine increases the expression of gasdermin D.

We observed a significant difference in GSDMD expression levels between the groups ($F_{(3,28)}$ = 52.228, *P*< 0.001). As it has been presented in Figure 4, Meth administration led to an increase in GSDMD levels in the Meth group compared to the saline group (2.34±0.15 vs 1.04 ± 0.051, *P*<0.001). In addition, melatonin treatment effectively reduced the increase in GSDMD levels in the Meth + melatonin group in comparison with the Meth group (1.66 ± 0.075 vs 2.34±0.15, *P* < 0.001).

Melatonin mitigated the inflammatory effects of Methamphetamine by reducing the levels of caspase-1

We observed significant changes in caspase-1 expression in the different experimental groups ($F_{(3,28)}$ = 86.219 , *P*<0.001) using Real-Time PCR analysis. Specifically, our data analysis showed that Meth injection resulted in increased caspase-1 levels in Meth in comparison with saline group (3.21 ± 0.18 vs 1.0±0.04, *P*<0.001, Figure

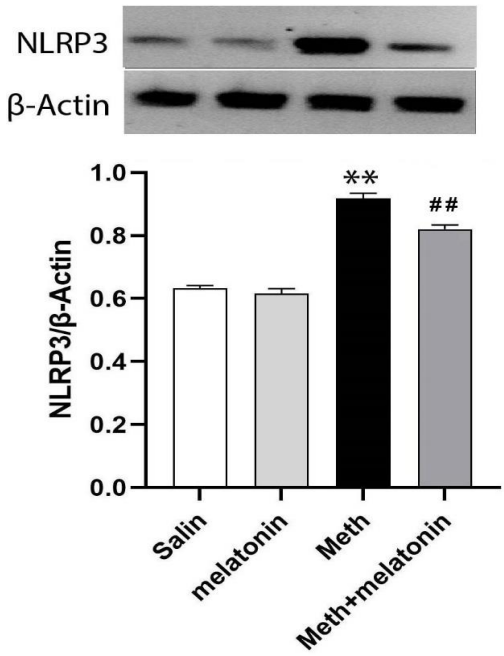

FIGURE 3. Western blot analysis showed that Meth use significantly increased NLRP3 levels in the hippocampus. Moreover, melatonin administration decreased NLRP3 in the hippocampus. **, $P< 0.001$ in comparison with the saline group; ##, $P< 0.001$ in comparison with the Meth group.

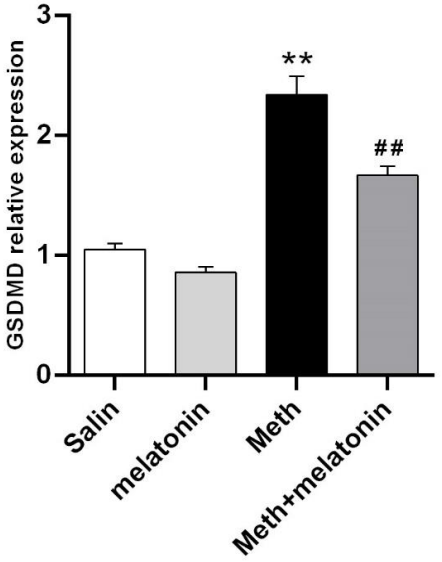

FIGURE 4. Gene expression analysis showed that Meth use significantly increased GSDMD levels in the hippocampus. Moreover, melatonin administration alleviated GSDMD levels. **, P< 0.001 in comparison with the saline group; ##, $P< 0.001$ in comparison with the Meth group.

5). Administration of melatonin effectively prevented the increase in caspase-1 levels in the Meth + melatonin group compared to the Meth group (2.22 ± 0.09 vs 3.21 ± 0.18, $P <0.001$). These results indicate that melatonin treatment successfully reduced Meth-induced increases in hippocampal caspase-1. This highlights the potential therapeutic role of melatonin in modulating caspase-1 expression and GSDMD and suggests its utility in reducing the neuroinflammatory responses associated with Meth exposure.

Discussion

The primary discoveries from this study can be summarized as (1) Administration of Meth to rat models resulted in neuroinflammation within the hippocampus, and (2) Conversely, melatonin was found to suppress both inflammation and pyroptosis. This study showed that melatonin treatment improved Meth-induced memory impairment by reducing the expression of NLRP3, IL-1β, caspase-1, and g GSDMD in the hippocampus. This process may be reflecting a promising strategy for dealing with Meth abuse. We observed significant memory impairment in the Meth group compared with the control group. These data are consistent with earlier findings on working memory impairment induced by Meth (Gaillard et al., 2021). In the present study, Meth injection increased hippocampal NLRP3, IL-1β, caspase-1, and GSDMD levels.

Various studies have shown the neurotoxicity of Meth in different areas of the brain (Jayanthi et al., 2021). The hippocampus is a complex and important structure for learning and memory (Rashidi et al., 2023). Any defects in the function of hippocampal neurons disrupt learning and memory. Previous studies have reported increased neuronal death in the hippocampus of Meth abusers (Sabrini et al., 2020). In line with the findings of our research, Golsarkhadan et al. reported that chronic administration of 5 mg/kg Meth impairs spatial memory and reduces long-term potentiation (LTP) in the hippocampus, and causes neuronal degeneration in the hippocampal tissue of rats. In addition, they observed that chronic Meth administration causes a significant decrease in the volume of the hippocampus along with gliosis enhancement (Golsorkhdan et al., 2020). In addition, it has been shown that Meth injection significantly reduces superoxide dismutase and glutathione peroxidase concentrations in the hippocampus, leading to increased oxidative stress. Interestingly, caspase-3 and apoptosis pathways

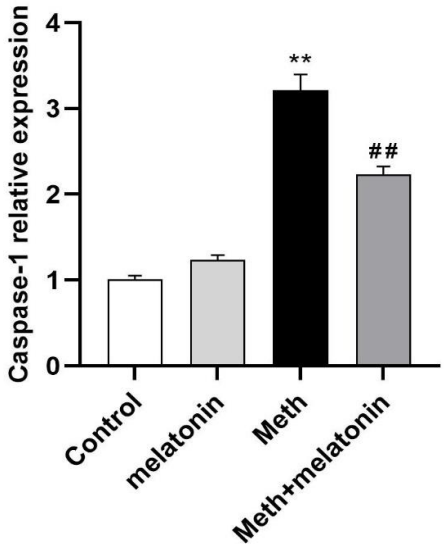

FIGURE 5. Gene expression analysis showed that Meth significantly increased caspase-1 levels in the hippocampus. Moreover, melatonin administration reduced caspase-1 levels. **, $P< 0.001$ in comparison with the saline group; ##, $P< 0.001$ in comparison with the Meth group.

increased in the hippocampus after Meth administration (Hadizadeh-Bazaz et al., 2021).

Our results showed a significant rise in NLRP3, IL-1β, caspase-1, and GSDMD levels after 3 weeks of Meth treatment. NLRP3, IL-1β, caspase-1, and GSDMD are expressed prominently in the hippocampus, prefrontal cortex, and certain regions of the brain that are prone to neurodegeneration (Pohlentz et al., 2022). Studies have shown that changes in NLRP3, IL-1β, caspase-1, and GSDMD expression levels are linked to the risk of anxiety and mood disorders (Roy et al., 2023). It has been seen that an increased level of NLRP3 causes a rise in the inflammatory cytokine expression in the hippocampus, which leads to memory impairment (Danielski et al., 2020). Consequently, it can be inferred that the memory impairment in the Meth-injected group may be partly related to an increase in hippocampal NLRP3, IL-1β, caspase-1, and GSDMD levels.

The pineal gland synthesizes a large amount of melatonin that is realized into the CSF (Dezfouli et al., 2019). Melatonin (10 mg/kg) was used in this study. It has been reported that 10 mg/kg of melatonin ameliorated Meth-induced cognitive impairment (Kraiwattanapirom et al., 2021). In addition, previous studies have demonstrated the neuroprotective and anti-inflammatory properties of 10 mg/kg melatonin (Kraiwattanapirom et al., 2021). The neuroprotective benefits of melatonin have been noted in various studies, even in the absence of melatonin membrane receptors such as MT1 and MT2 (Slominski et al., 2012). For example, Kilic et al. demonstrated significant neuroprotective effects of melatonin against cerebral ischemia in mice lacking MT1 and MT2 receptors(Kilic et al., 2012), suggesting that these membrane receptors may not be necessary for the neuroprotective effects of melatonin. In a study, it was found that administration of melatonin protects hippocampal neurons against Meth and reduces the neurotoxic effects of Meth on working memory and anxiety (Panmak et al., 2021).

Our findings also support the neuroprotective effects of melatonin on memory following Meth injection. Melatonin has been shown to reduce oxidative damage in the central nervous system, reduce inflammation, and improve learning and memory in rat models (Dezfouli et al., 2019). Interestingly, melatonin has been shown to reduce Meth induced neurodegeneration, enhance neurosurvival pathways, and preserve mitochondrial integrity (Parameyong et al., 2013). Here, we observed that melatonin administration reduced NLRP3, IL-1β, caspase-1, and GSDMD in the hippocampus. Consistent with our result, Arioz et al. evaluated the effects of melatonin on behavioral changes and expression of inflammatory cytokines in the hippocampus of rats in LPS-induced behavioral disorders, as well as its effects on NLRP3 inflammasome activation, oxidative stress, and pyroptotic cell death in rat microglia. Their results

showed that melatonin inhibits LPS and NLRP3 inflammasome activation in mouse microglia in vitro, which inhibits NLRP3 expression, and IL-1β secretion. In addition, melatonin prevented pyroptosis and the production of ROS. They reported that the beneficial effects of melatonin are dependent on SIRT1-mediated NLRP3 inflammasome activation and these neuroprotective effects were suppressed by SIRT1 inhibitor treatment(Arioz et al., 2021).

In this study, the rat model with chronic Meth use has shown higher levels of DSDMD and NLRP3 in the hippocampus, which indicates pyroptosis. Meth can induce GSDME-induced pyroptosis in rat hippocampus. It has been found that Meth-induced neurotoxicity is achieved through the NLRP3/caspase-1/ GSDMD signaling pathway and the use of modulators of this complex can significantly prevent Meth-induced cognitive and memory disorders in rats. Strategies that modulate pyroptosis may reduce Meth-induced damage to hippocampal neurons.

Conclusion

This study showed that melatonin administration improved working memory and reduced inflammasome formation and pyroptosis pathway in Meth-injected rats. Here, we propose that melatonin neuroprotection is partially related to inflammatory signaling in the central nervous system. This mechanism has important implications to pave the way for the new strategies against Meth abuse.

Acknowledgment

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Conflict of interests

All authors declare that there is no conflict of interest.

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