



Melatonin Mitigates Central Sensitization and Nociceptive Pain in Spinal Cord and Dorsal Root Ganglia of FM Rat Model: Modulation of SIRT1/PGC-1 α /MAPK/NF- κ B Signaling

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Abstract

Fibromyalgia (FM) is a central sensitization syndrome characterized by neuroinflammation and synaptic hyperexcitability, which amplifies pain signals in the spinal cord (SC) and dorsal root ganglia (DRG). Melatonin (MEL) has demonstrated analgesic and antinociceptive properties in experimental models, supporting its clinical application in various pathological conditions. This study aimed to elucidate the impact of Melatonin on neuroinflammation and glutamatergic dysregulation in the spinal cord and DRG using a Reserpine-induced fibromyalgia model (RIFM). RIFM was induced in female rats by administering Reserpine (1 mg/kg/day, s.c.) for three consecutive days. Melatonin (10 mg/kg, orally) was administered for three days following the peak of pain on day 7. Melatonin significantly ameliorated stimulus-evoked pain in thermal and mechanical tests, with a resolution of spontaneous pain noted by the rat grimace scale. Moreover, motor activity in the open field task and depressive behavior in the tail suspension test (TST) were alleviated following Melatonin administration. Fibromyalgia pain was associated with enhanced glutamatergic transmission, as shown by immunohistochemical assessment of synaptophysin and PSD95 in the DRG along with VGLUT, PSD95, NMDA, NMDA receptor 2B, and AMPA in the spinal cord. Reserpine-induced disturbances in mitochondrial biogenesis markers, SIRT1 and PGC-1 α , were followed by an upsurge of TNF- α , NF κ B, and P38-MAPK. This neuroinflammatory milieu was marked by elevated ionized calcium-binding adaptor molecule-1 (Iba-1) in activated microglia within the spinal cord. These pathological findings were notably mitigated by Melatonin, as reflected by increased expressions of spinal MT1 and MT2 receptors. In conclusion, Melatonin exhibited antinociceptive and anti-inflammatory effects by modulating glutamate neurotransmission, mitochondrial dysfunction, and microglial activation, thereby alleviating nociceptive pain in the experimental RIFM.

Keywords Fibromyalgia · Melatonin · Glutamatergic dysregulation · Dorsal root ganglia · Spinal cord · Mitochondria

Introduction

Fibromyalgia (FM) syndrome, the most common central sensitivity syndrome (CSS), affects approximately 2–5% of the population (Boomershine 2015; Sarzi-Puttini et al. 2020). It is a persistent disease that is distinguished by severe pain in the muscles and joints, with mechanical and thermal allodynia being common among FM patients. FM typically occurs in middle-aged women at a ratio of 3:1, although it can affect both sexes at any age (Brum et al. 2020). There are multiple contributing factors, such as central sensitization and neuroinflammation mediated by cytokines and glial activation. Additional factors include autonomic dysregulation, small fiber neuropathy, as well genetic and epigenetic predispositions such as polymorphisms in the

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catechol-O-methyltransferase well as alterations in pain processing pathways (Siracusa et al. 2021; Gyorfı et al. 2022).

In recent years, the International Association for the Study of Pain has acknowledged “nociplastic pain” as a tertiary mode of pain genesis. When there is no actual or anticipated tissue injury, nociplastic pain is defined as pain that results from altered nociception (Fitzcharles et al. 2021). Excitatory neurotransmitters like glutamate facilitate synaptic transmission within the dorsal horn and dorsal root ganglia (DRG), amplifying pain transmission through ascending pathways conveying nociceptive signals from the periphery to the brain (Latremoliere and Woolf 2009; Yam et al. 2018). Independent reviews confirm that mitochondrial dysfunction, activated glutamate receptors, and microglial activation in the spinal dorsal horn synergistically enhance ascending nociceptive signaling (Kung et al. 2013; Deng et al. 2019; Cao et al. 2024; Espinoza and Papadopoulos 2025). Additionally, the proximity of the DRG to the subarachnoid space may link central sensitization to the elevated levels of the pain-promoting neurotransmitter glutamate in the cerebrospinal fluid of FM patients, as observed through neuroimaging of the brain (Martínez-Lavín 2022).

Mitochondria play a vital role at the cellular level in fibromyalgia (FM) patients (Meeus et al. 2013; Brum et al. 2020; Macchi et al. 2024). In this context, mitochondrial dysfunction refers to impaired biogenesis and activity, often characterized by reduced expression of key regulatory proteins such as phosphorylated peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α) and Sirtuin 1 (SIRT1), both of which are essential for maintaining mitochondrial health. Evidence from clinical and pre-clinical studies indicates that mitochondrial dysfunction contributes to increased pain sensitivity (Brum et al. 2020; Jung et al. 2021). Moreover, mitochondrial dysregulation frequently triggers an inflammatory response through activation of pathways such as NF- κ B and P38 MAPK, thereby exacerbating the neuroinflammatory environment (Qin et al. 2018; Castro et al. 2022). These molecular alterations foster neuroinflammation and facilitate pain sensitization in FM. The resulting disturbed environment promotes microglial activation, which is associated with glutamate release in the spinal dorsal horn, further amplifying central sensitization (Kawasaki et al. 2008; Domercq et al. 2013; Ji et al. 2018; Chen et al. 2018; Gu et al. 2022; Atta et al. 2023a; Kohno and Tsuda 2025). Microglial activation, a hallmark of neuroinflammation, can be identified by increased expression of ionized calcium-binding adapter molecule 1 (Iba-1), a calcium-binding protein specifically expressed in microglia with surge of proinflammatory cytokines (Gui et al. 2016; Qi et al. 2016; Vanderwall and Milligan 2019; Zhang et al. 2025). Thus, regulating mitochondrial function may suppress neuroinflammation by modulating microglial

activation and glutamatergic dysregulation. In addition to DRG, the spinal dorsal horn showed glutamatergic changes in chronic pain through enhanced excitatory neurotransmission, viz., glutamatergic signaling. Upregulation of markers such as vesicular glutamate transporter (VGLUT), post-synaptic density protein 95 (PSD95), and ionotropic glutamate receptors (NMDA and AMPA) contributes to synaptic potentiation and central sensitization, critical for persistent pain (Liu and Salter 2010; Niciu et al. 2012; Bardoni 2013; Turan Yücel et al. 2023; Jang and Garraway 2024). Therefore, there is an urgent need for therapeutic interventions targeting mitochondrial dysfunction and synaptic hyperexcitability in FM patients.

Melatonin is a neurohormone that shows antinociceptive effects across various pain models. Melatonin exerts its actions primarily through its receptor subtypes, MT1 and MT2, which are expressed in the spinal cord (Gao et al. 2020; Xiong et al. 2024). These receptors have been extensively investigated in models of neuropathic pain, migraine, and irritable bowel syndrome (Srinivasan et al. 2012). Melatonin attenuated mitochondrial dysfunction and neuroinflammation via its receptors and downstream signaling pathways, including SIRT1 activation (Zeng et al. 2023). Moreover, SIRT1 mediated Melatonin’s inhibitory effects on microglial activation, further reducing neuroinflammation (Merlo et al. 2020). In models of nerve injury, Melatonin suppressed microglial activation via inhibiting the p38 MAPK signaling pathway that modulated behavioral hypersensitivity (Chiang et al. 2013). However, a gap remains in exploring Melatonin’s impact on mitochondrial dysfunction and neuroinflammation-induced central sensitization in FM.

Therefore, the present study aimed to examine melatonin’s potential to alleviate central sensitization and nociplastic pain in a reserpine-induced FM model by restoring mitochondrial function and attenuating the consequent neuroinflammatory and glutamatergic disturbances in the spinal cord and DRG. As well, investigating changes in MT1 and MT2 receptor expressions that provide insights into the mechanisms underlying Melatonin’s potential therapeutic effects in FM. To achieve this aim, FM was induced using reserpine (RES). The RES-induced fibromyalgia model (RIFM) is a well-established and extensively used animal model that replicates the etiological and clinical features of FM, encompassing (i) the cardinal symptoms experienced by FM patients, such as musculoskeletal pain, spontaneous nociception, and pain hypersensitivity; (ii) primary comorbidities, including fatigue, depression, sleep disturbances, and anxiety; and (iii) characteristic pathological mechanisms, such as peripheral and central sensitization, neuroinflammation, and alterations in the levels of excitatory and inhibitory neurotransmitters (Yao et al. 2020; Brum et al. 2022).

Materials and methods

Animals

Female Wistar rodents (160–200 g) were obtained from the animal facility of the Egyptian Drug Authority in Giza, Egypt. The study was designed to focus on female rats intentionally, as fibromyalgia (FM) affects women at a significantly higher rate, with approximately 80–96% of FM patients being female (Wolfe et al. 2010; Carson et al. 2010; Cabo-Meseguer et al. 2017; Ruschak et al. 2023). Before the experiment, the animals were subjected to laboratory conditions at the Faculty of Pharmacy, Cairo University's animal facility, for a period of seven days. The rodents were confined in conventional polycarbonate enclosures (40 × 25 × 15 cm) in groups of five during the acclimatization and experimental phases. The environmental conditions were meticulously monitored, with a stable room temperature of 25 ± 2 °C, a 12-hour light/dark cycle (lights on from 7:00 AM to 7:00 PM), and a relative humidity of $60 \pm 10\%$. In an effort to observe the rodents without substantially disrupting their nocturnal period, the lighting was meticulously controlled with faint red lights. In order to guarantee sanitary conditions, bedding was replaced daily, and cages were cleansed. The animals were provided with unlimited access to potable water and standard food pellets (National Research Centre diet, Giza, Egypt) during the course of the investigation. Furthermore, daily monitoring of room humidity and ambient temperature was implemented to mitigate environmental fluctuations that could potentially affect the experimental outcomes.

Results

MEL Alleviated Spontaneous- and Evoked-Pain Perception in the FM-Like Model

To assess whether Melatonin affects spontaneous pain — a key characteristic of FM — spontaneous facial expressions were recorded using the rat grimace scale, which was then quantified to measure spontaneous pain. Five major changes regarding the rat grimace scale were represented in Fig. 1(I): (A) facial expression-captured photos of different groups, (B) orbital tightening, (C) nose/cheek flattening, (D) ear changes, and (E) whisker changes. At $p < 0.0001$, the FM group revealed (A) eye closure or eye squeezing, (B) less bulging of the nose and cheek, (C) increased ear folding, curling, and outward angling, resulting in a pointed shape, and (D) whiskers moved forward away from the face, tending to appear as if standing on end, to reach six folds [$F_{(2,27)} = 21.58$], 8.5 folds [$F_{(2,27)} = 23.05$], six folds

[$F_{(2,27)} = 20.88$], and 4.75 folds [$F_{(2,27)} = 35.53$], respectively, compared to the naïve rats.

In contrast, the treated group verified the substantial efficacy of Melatonin in reducing spontaneous pain, as reflected by (A) orbital tightening, (C) ear, and (D) whisker changes reaching the normal control scores. Meanwhile, FM rats treated with Melatonin only normalized (B) nose and cheek flattening by 47% compared to the FM group.

To evaluate the effect of Melatonin administration on mechanical and thermal pain sensitivity in RIFM, the study employed a series of behavioral assessments, such as the von Frey filament test, cold allodynia test, hot plate test, Randall-Sellito test, and tail suspension test, to comprehensively characterize pain responses associated with FM. These tests were chosen to measure different modalities of pain sensitivity, thereby providing a detailed understanding of Melatonin's potential anti-nociceptive effects. Figure 1(II) demonstrates rats with FM induced by Reserpine presented an exacerbation in the urged pain response, as noted by the aberrations in the nociceptive reflexes through the decremented threshold of (A) the von Frey test (VFT) and (B) CAPWL, reaching 71% [$F_{(2,27)} = 6.376$, $p = 0.0053$, $\eta^2 = 0.321$] and 40% [$F_{(2,27)} = 20.12$, $p = 0.0003$, $\eta^2 = 0.770$], respectively, compared to the normal rats.

Furthermore, using the Randall-Sellito and hot plate tests, FM rats manifested a marked hindrance in the (C) Randall-Sellito mechanical threshold (RSMT) and (D) hot plate reaction latency (HPRL) by 71% [$F_{(2,27)} = 10.06$, $p = 0.0021$, $\eta^2 = 0.626$] and 54% [$F_{(2,27)} = 6.921$, $p = 0.0068$, $\eta^2 = 0.339$], respectively, relative to the healthy rats. Furthermore, a supraspinal pain response was revealed in the reserpinized rats, indicated by extended immobility time in (E) TST, reaching 2.27-fold [$F_{(2,27)} = 18.91$, $p = 0.0001$, $\eta^2 = 0.583$] compared to the vehicle group. Conversely, oral consumption of Melatonin for three days significantly boosted the withdrawal threshold to attain 1.3-fold and 2.3-fold in (A) VFT ($p = 0.0412$) and (B) CAPWL ($p = 0.0006$), respectively, unlike the FM group values. Additionally, Melatonin's anti-allodynic effect was verified by increased pain endurance in (C) RSMT and (D) HPRL, achieving 2.48-fold and 2.08-fold relative to the Reserpine-induced FM rats. Ultimately, Melatonin treatment mitigated the pain and improved mobility to approach 56% relative to the untreated rats, at $p = 0.0003$. All these results are summarized in Fig. 1 (I) and (II), reflecting Melatonin's antinociceptive effect.

Melatonin Restored Motor Coordination in the FM-like Model

To investigate whether Melatonin can modulate motor disturbances associated with the FM model, the Rota rod

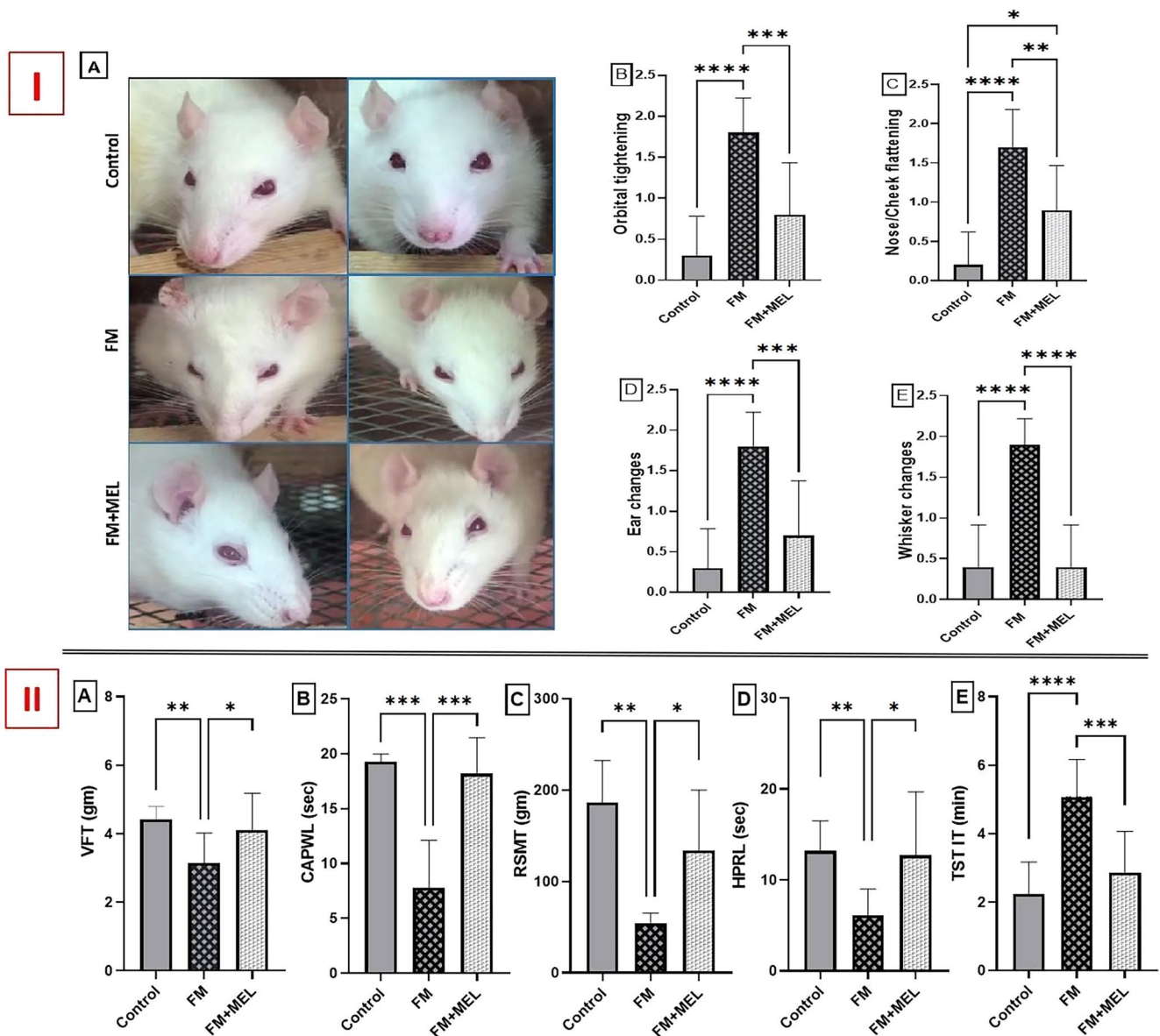


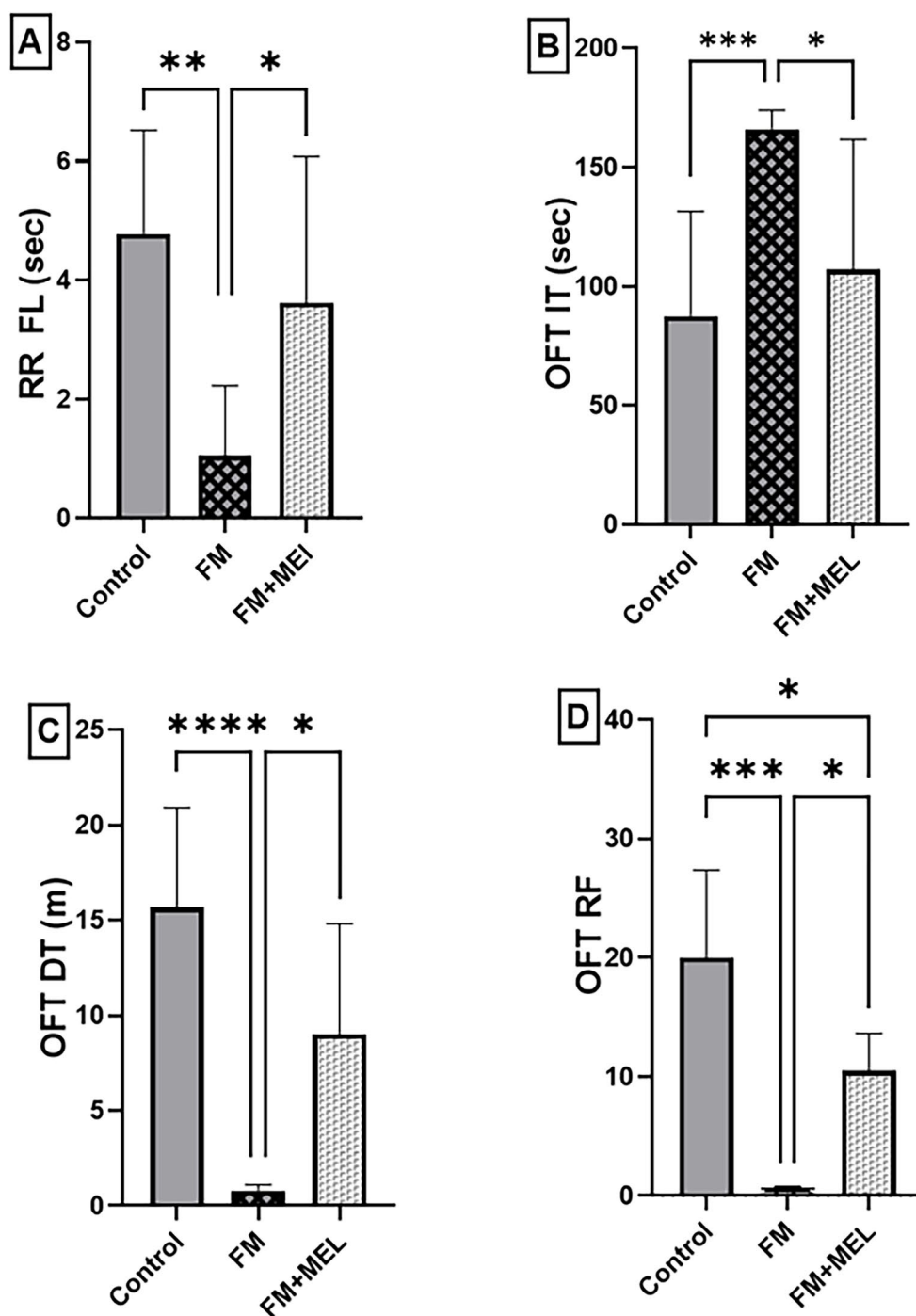
Fig. 1 (I) Impact of Melatonin (10 mg/kg, oral, 3 days) on (A) facial expression-captured photos, (B) orbital tightening, (C) nose/cheek flattening, (D) ear changes, and (E) whisker changes tracking spontaneous pain in the FM-like model using the rat grimace scale. Using one-way ANOVA and Tukey's post-hoc test ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$), the mean \pm SD of rats ($n = 10$) per group is indicated by each bar with a vertical line. **(II)** Impact of Melatonin (10 mg/kg, oral, 3 days) on FM rats using (A) VFT, (B) CAPWL, (C) RSMT, (D) HPRL, and (E) TST IT to measure nociceptive reflexes

and hyperalgesic behaviors in response to the induced pain. Using one-way ANOVA and Tukey's post-hoc test ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$), the mean \pm SD of rats ($n = 10$) per group is indicated by each bar with a vertical line. VFT: von Frey test; CAPWL: cold allodynia paw withdrawal latency; FM: fibromyalgia model; HPRL: hot plate reaction latency; MEL: Melatonin; RSMT: Randall-Sellitto mechanical threshold; TST IT: tail suspension test immobility time

test and open-field test were employed. These assessments aimed to evaluate potential improvements in motor coordination and activity levels, thereby exploring additional therapeutic effects of Melatonin beyond pain modulation (Fig. 2). In the Rota rod test, Reserpine-induced FM drastically decreased (A) the rat's retention time on the RR by 78% [$F_{(2,27)} = 8.312$, $p = 0.0016$, $\eta^2 = 0.41$] compared to the

healthy rats. Regarding the open field test, FM rats revealed a substantial reduction in (B) open field test immobility time (OFT IT) by 90% [$F_{(2,27)} = 11.64$, $p = 0.0002$, $\eta^2 = 0.492$] and (C) open field test distance traveled (OFT DT) by 95% [$F_{(2,27)} = 23.03$, $p < 0.0001$, $\eta^2 = 0.767$], contrasted with healthy ones. In addition, the subcutaneous administration of RES effectively aggravated the neuropathic pain reported

Fig. 2 Impact of Melatonin (10 mg/kg, oral, 3 days) on locomotor activity and motor coordination by (A) RRFL using the Rota rod test and (B) OFT IT, (C) OFT DT, and (D) OFT RF using the open field test. Using one-way ANOVA and Tukey's post-hoc test ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$), the mean \pm SD of rats ($n = 10$) per group is indicated by each bar with a vertical line. FM: fibromyalgia model, MEL: Melatonin, OFT DT: distance traveled in open field test, OFT IT: immobility time in open field test, OFT RF: rearing frequencies in open field test, RR FL: falling latency in the Rota rod test



herein by reducing the (D) rearing frequencies in the open field test (OFT RF) in the FM group by 99% [$F_{(2,27)} = 22.87$, $p = 0.0001$, $\eta^2 = 0.821$]. Contrarily, Melatonin prolonged (A) RIFM's falling latency by 77% ($p = 0.0279$) and effectively countered the motor alterations manifested by reserpinized rats in the OFT, resulting in 0.65-fold for OFT IT ($p = 0.0169$), 11.46-fold for OFT DT ($p = 0.0104$), and a notable rise in vertical upright behavior by 51.5-fold ($p = 0.0143$).

Melatonin Mitigated Histological Alterations in the DRG Within the FM-like Model

Normal control samples exhibited intact neuronal types of various sizes with preserved subcellular details (black arrow), few sporadic degenerated neurons, well-organized satellite ganglionic cells (yellow arrowhead), and Schwann cells with intact myelinated nerve fibers. In contrast, FM samples showed focal areas of degenerated and shrunken

neurons with nuclear pyknosis and occasional chromatolysis (red arrow), accompanied by wider interneuronal spaces and a significant loss of perineuronal ganglionic satellite cells. However, FM+Melatonin rats demonstrated neuroprotective efficacy, as evidenced by an increased number of intact neurons (black arrow) similar to those in normal control samples, moderate restoration of satellite ganglionic cell densities (yellow arrowhead), and a reduced extent of nerve damage (red arrows) (Fig. 3).

Melatonin up-regulated the MT1R and the MT2R in the Spinal Cord of RIFM

To explore the role of Melatonin receptors in mediating its anti-nociceptive effects, gene expression levels of MT1R and MT2R in the rat FM spinal cord were measured using quantitative polymerase chain reaction. This analysis aimed to determine whether alterations in receptor expression contribute to Melatonin's antinociceptive properties. Reserpine administration drastically reduced MT1R ($F_{(2,6)}=27.99$, $p=0.0009$, $\eta^2=0.903$) and MT2R ($F_{(2,6)}=245.3$, $p<0.0001$, $\eta^2=0.988$) in the spinal cord by 72% and 67%, respectively, compared to normal mRNA expression in healthy rats. Melatonin significantly increased MT1R and MT2R mRNA expression in treated animals to 70% and 80% of the FM group, as shown in Fig. 4 A and B.

Melatonin Ameliorated Mitochondrial Dysfunction in the Spinal Cord of RIFM

To investigate whether Melatonin influences mitochondrial biogenesis in FM, the expression levels of SIRT-1 and PGC-1 α were measured. These markers were selected to evaluate potential mitochondrial effects of Melatonin in the context of FM. Dysfunction in mitochondrial biogenesis was confirmed in FM rats through a reduction in the spinal cord (C) Sirt1 protein expression by 68% ($F_{(2,6)}=265.3$, $p<0.0001$, $\eta^2=0.989$)

and (D) PGC1 α protein content by 67% ($F_{(2,15)}=218.5$, $p<0.0001$, $\eta^2=0.967$), as shown in Fig. 4. Melatonin treatment resulted in an increase in (C) Sirt1 protein expression (2.5-fold) and (D) PGC1 α protein content (2.28-fold) compared to the FM group (Fig. 4 C and D).

Melatonin Restrained Neuroinflammatory Mediators, MAPK and NF- κ B, in the Spinal Cord of RIFM

To evaluate the anti-neuroinflammatory effects of Melatonin, the levels of key neuroinflammatory mediators, p-MAPK and NF- κ B, were measured. These markers help determine the extent to which Melatonin modulates neuroinflammation in FM. As depicted in Fig. 5A, phosphorylation of MAPK protein expression significantly increased in FM rats, reaching 5.78-fold [$F_{(2,6)}=56.53$, $p=0.0001$, $\eta^2=0.949$] compared to the healthy group. Furthermore, Fig. 5B shows that FM rats exhibited a 2.7-fold increase in NF- κ B levels [$F_{(2,15)}=6357$, $p<0.0001$, $\eta^2=0.998$] compared to naïve rats. Conversely, Melatonin oral treatment inhibited p38MAPK phosphorylation, reducing it by 38% compared to reserpinized rats. Additionally, Melatonin-treated rats showed a reversal effect on NF- κ B levels, decreasing them by 42.5% compared to untreated FM rats.

Melatonin Repressed the Neuroinflammatory Executors in the Spinal Cord of RIFM

The ability of Melatonin to modulate key neuroinflammatory mediators in the FM model was evaluated by measuring the levels of cytokines, specifically TNF- α and IL-1 β . These cytokines are critical indicators of neuroinflammation and help to assess Melatonin's therapeutic potential. In Fig. 5, s.c. Reserpine injection induced neuroinflammation in the rat spinal cord, as evidenced by increased protein levels of (A*) TNF- α (3.53-fold) [$F_{(2,15)}=139.8$, $p<0.0001$,

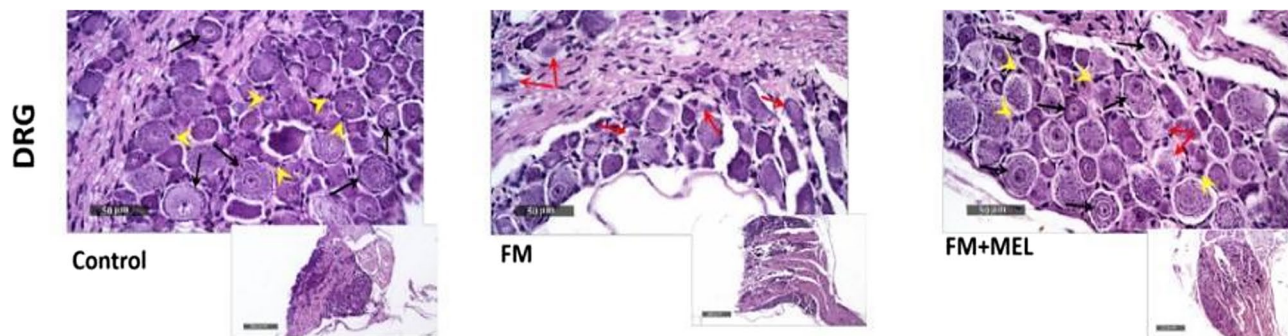


Fig. 3 Impact of Melatonin (10 mg/kg, oral, 3 days) on histopathological changes in the FM rat model. Photomicrographs of H&E staining of the DRG are shown. Black arrows indicate intact, well-organized neurons with preserved subcellular details; the yellow arrowhead highlights sporadic degenerated neurons, well-organized satellite gangli-

onic cells, and Schwann cells exhibiting intact myelinated nerve fibers. The red arrow points to degenerated neurons with nuclear pyknosis, occasional chromatolysis, wider interneuronal spaces, and marked perineuronal ganglionic satellite cell loss. DRG: dorsal root ganglia, FM: fibromyalgia, MEL: Melatonin. Scale bar: 200 and 50 μ m

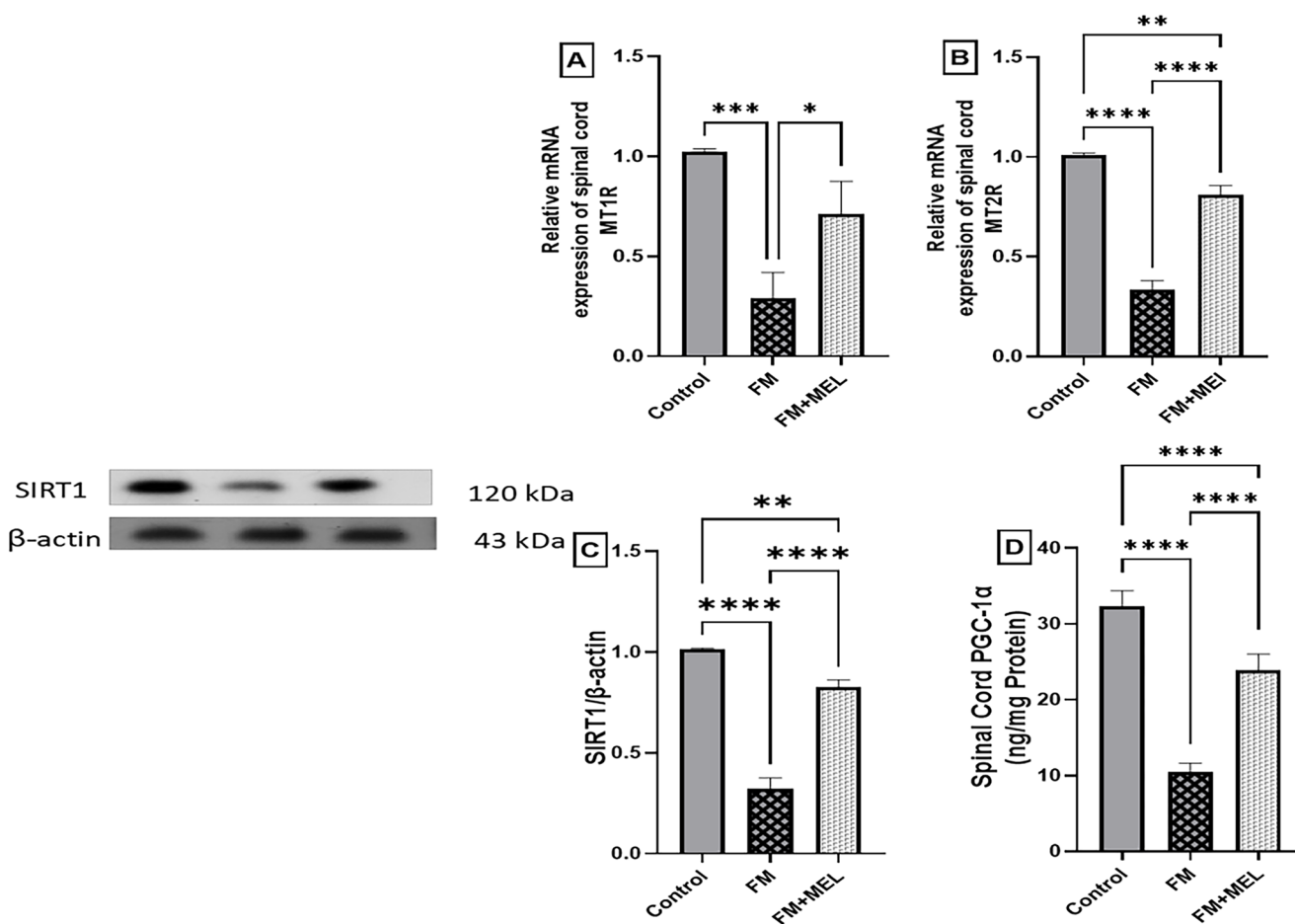


Fig. 4 Impact of Melatonin (10 mg/kg, oral, 3 days) on mRNA expression of the spinal cord (A) MT1R and (B) MT2R in the FM-like model. Using one-way ANOVA and Tukey's post-hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$), the mean \pm SD of rats ($n = 3$) per group is indicated by each bar with a vertical line. MEL: Melatonin, FM: fibromyalgia model, MT1R: Melatonin receptor 1, and MT2R: Melatonin receptor 2. In addition to the impact of Melatonin on the spinal cord, (C) SIRT1 protein expression and (D) PGC1 α

protein content indicate mitochondrial biogenesis in the FM-like model. The mean \pm SD of rats ($n = 3$ for SIRT1 and $n = 6$ for PGC1 α per group) is represented by each bar with a vertical line. Analyses were conducted using one-way ANOVA and Tukey's post-hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$). FM: fibromyalgia model, MEL: Melatonin, PGC1 α : peroxisome proliferator-activated receptor gamma coactivator 1-alpha, SIRT1: silent information regulator sirtuin 1

$\eta^2 = 0.949$], (B*) IL-1 β (2.75-fold) [$F_{(2,15)} = 1748, p < 0.0001, \eta^2 = 0.996$], and (C*) IL-6 (2.85-fold) [$F_{(2,15)} = 654.9, p < 0.0001, \eta^2 = 0.989$] compared to naïve control rats (Fig. 5). Melatonin-treated rats attenuated this increase, reducing TNF- α , IL-1 β , and IL-6 levels by 47%, 46%, and 47%, respectively, compared to diseased rats.

Melatonin Inhibited the Up-Regulation of the Spinal Microglial Iba-1 Protein Contents and NMDA Receptor Subunit 2B (NR2B) in the Spinal Cord of RIFM

To study Melatonin's efficacy on glutamate dysregulation—identified as a key factor in pain hypersensitivity in the FM model—changes in glutamate receptor NR2B

protein content were measured. Additionally, the coincidence between glutamate dysregulation and microglial activation was assessed by evaluating the activated microglial marker Iba-1. An upregulation of Iba1 protein content was detected by ELISA following Reserpine injections, showing a 139% increase [$F_{(2,15)} = 2034, p < 0.0001, \eta^2 = 0.996$] compared to control rats. The Melatonin group significantly reduced SC Iba1 levels to 45% relative to the induction group (Fig. 6A). As shown in Fig. 6B, reserpinized rats exhibited a 2.83-fold increase in NR2B expression [$F_{(2,15)} = 176.5, p < 0.0001, \eta^2 = 0.96$] compared to the normal group. However, Melatonin-treated rats significantly attenuated this NR2B upregulation by 58% compared to reserpinized rats.

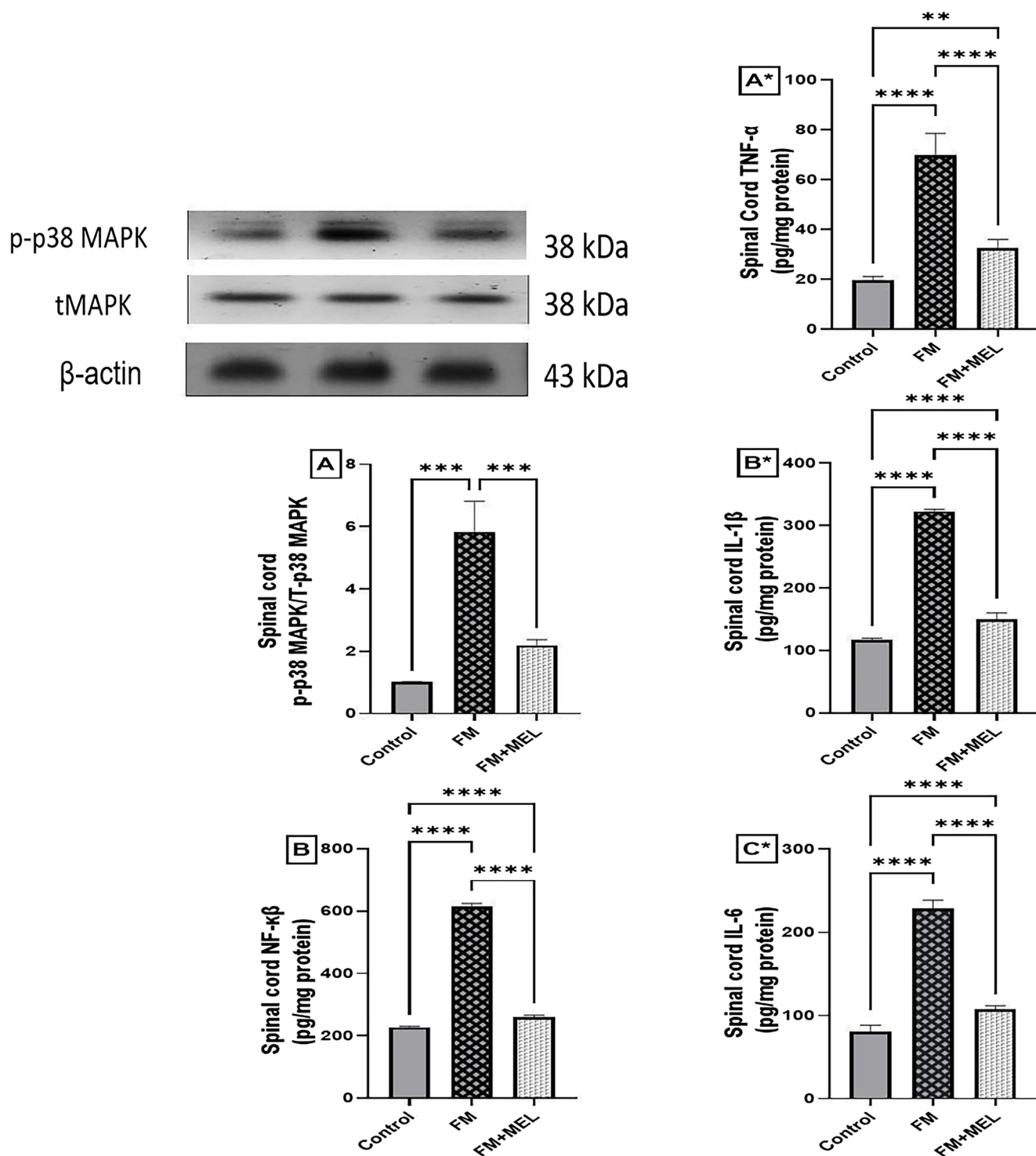
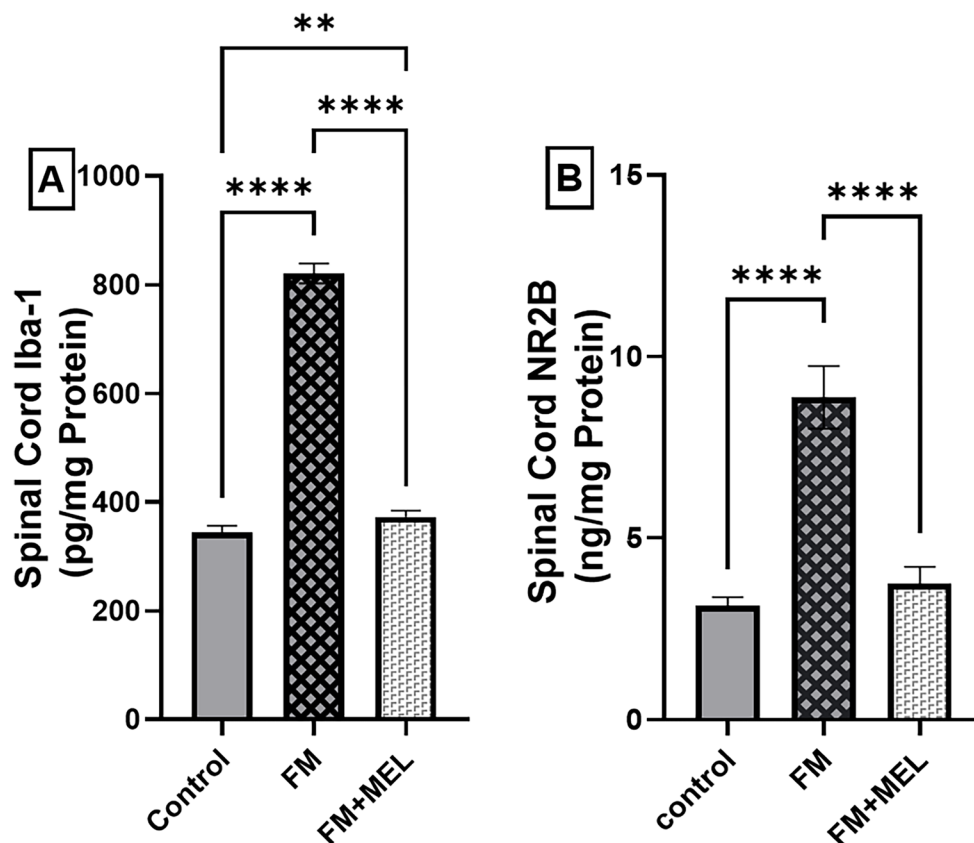


Fig. 5 Impact of Melatonin (10 mg/kg, oral, 3 days) on spinal cord pro-inflammatory modulators (A) p-p38 MAPK/total p38-MAPK and (B) NF-κB in the FM-like model. Using one-way ANOVA and Tukey's post-hoc test ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$), the mean \pm SD of rats ($n = 3$ for MAPK and $n = 6$ per group for NF-κB) is represented by each bar with a vertical line. FM: fibromyalgia model, MAPK: mitogen-activated protein kinases, MEL: Melatonin, NF-κB: nuclear factor kappa B. Impact of Melatonin (10 mg/kg, oral,

3 days) on spinal cord pro-inflammatory cytokines (A*) TNF-α, (B*) IL-1β, and (C*) IL-6 in the FM-like model. Using one-way ANOVA and Tukey's post-hoc test ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$), the mean \pm SD of rodents ($n = 6$) per group is indicated by each bar with a vertical line. FM: fibromyalgia model, IL-1β: interleukin-1 beta, IL-6: interleukin-6, MEL: Melatonin, TNF-α: tumor necrosis factor

Fig. 6 Impact of Melatonin (10 mg/kg, oral, 3 days) on spinal cord (A) Iba1 and (B) NR2B protein content in the FM-like model. Using one-way ANOVA and Tukey's post-hoc test ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$), the mean \pm SD of rodents ($n = 6$) per group is indicated by each bar with a vertical line. FM: fibromyalgia model, Iba1: ionized calcium-binding adaptor molecule 1, MEL: Melatonin, NR2B: NMDA receptor subunit 2B



Melatonin Attenuated the Elevated Levels of Glutamatergic-Related Synaptic Markers: VGLUT, PSD95, NMDA, and AMPA in the Spinal Dorsal Horn in the FM-like Model

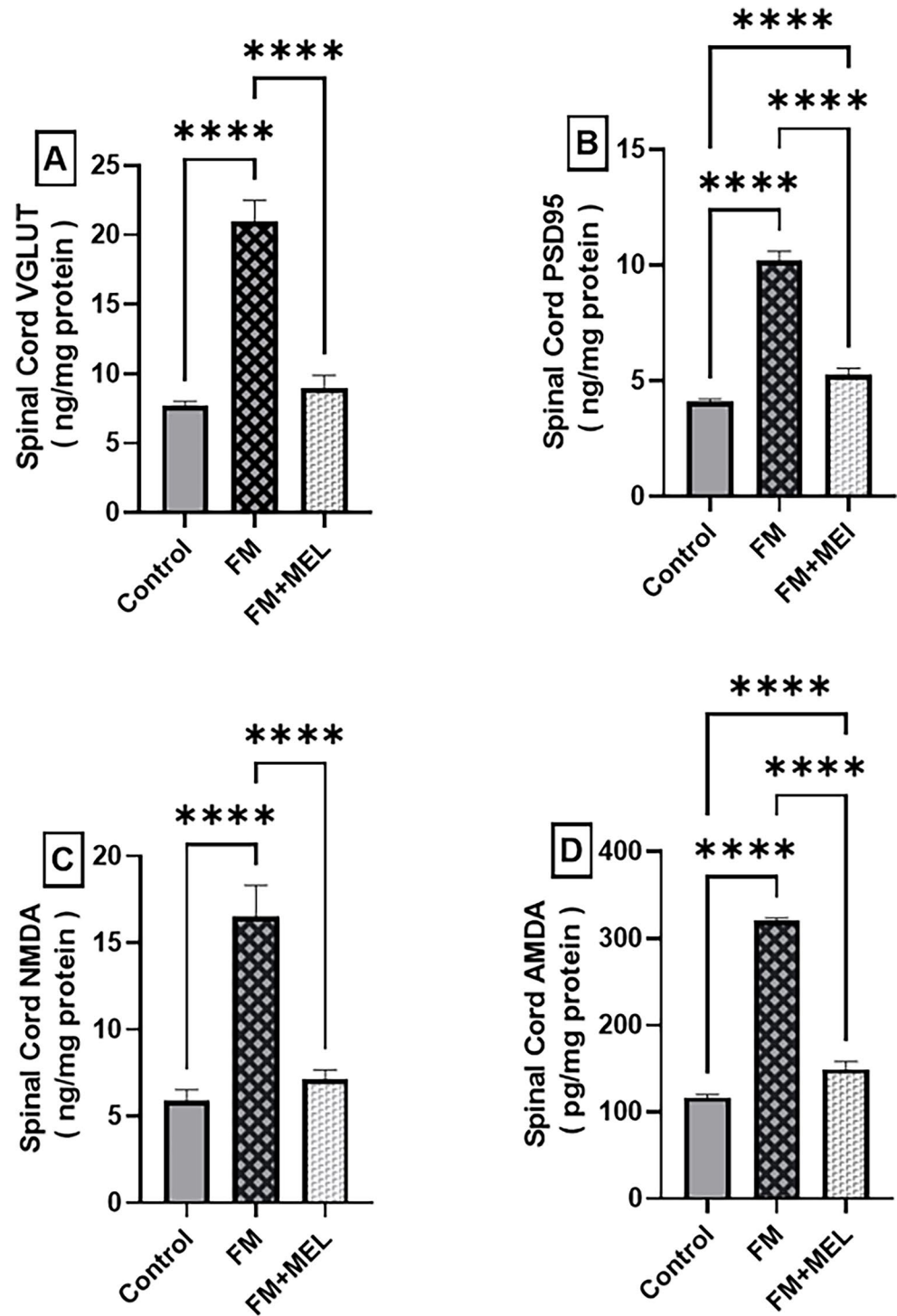
To determine if Melatonin influences glutamatergic changes in chronic pain, the study evaluated glutamatergic pre- and postsynaptic markers, VGLUT, PSD95, and ionotropic glutamate receptors; NMDA and AMPA in the spinal dorsal horn, a key area for synaptic potentiation and central sensitization in persistent pain. At $p < 0.0001$, the FM group revealed elevated protein levels of all 4 markers, VGLUT, PSD-95, NMDA, and AMPA, reaching 2.7-fold [$F(2,15) = 282.4$, $\eta^2 = 0.974$], 2.5-fold [$F(2,15) = 749.1$, $\eta^2 = 0.991$], 2.8-fold [$F(2,15) = 156$, $\eta^2 = 0.953$], and 4.75-fold [$F(2,27) = 35.53$], respectively, compared to the naïve rats. Melatonin treatment significantly reduced glutamatergic alterations in the FM spinal dorsal horn. (A) VGLUT and (C) NMDA protein levels were normalized to 43% of control values compared to the untreated group. (B) PSD-95 and (D) AMPA protein levels were also reduced by 48% and

53%, respectively, compared to the FM group after Melatonin treatment (Fig. 7).

Melatonin Attenuated the Elevated Levels of Glutamatergic-Related Synaptic Markers, PSD-95 and Synaptophysin in the DRG in the FM-like Model

To study the influence of Melatonin on glutamatergic-related synaptic markers; PSD95 and synaptophysin in the DRG were evaluated. PSD95 is a postsynaptic density protein associated with glutamatergic synapses that assess postsynaptic receptor clustering and synaptic strength. Synaptophysin is a presynaptic vesicle protein that marks synaptic density and activity, including glutamatergic terminals. FM animals exhibited overexpression of PSD-95 and synaptophysin in the DRG compared to normal animals, reaching 2.1-fold [$F(2,6) = 69.16$, $p < 0.0001$, $\eta^2 = 0.96$] and 1.75-fold [$F(2,6) = 28.37$, $p = 0.0009$, $\eta^2 = 0.904$], respectively. Conversely, Melatonin-treated rats showed a 69% reduction in both PSD-95 and synaptophysin compared to the untreated group (Fig. 8 A and B).

Fig. 7 Impact of Melatonin (10 mg/kg, oral, 3 days) on spinal cord (A) VGLUT, (B) PSD95, (C) NMDA, and (D) AMPA protein content in the FM-like model. Using one-way ANOVA and Tukey's post-hoc test ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$), the mean \pm SD of rodents ($n = 6$) per group is indicated by each bar with a vertical line. AMPA receptor: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor, FM: Fibromyalgia, MEL: Melatonin, NMDA: N-methyl-D-aspartate receptors, PSD95: Postsynaptic density protein 95, VGLUT: Vesicular glutamate transporter



Discussion

This study demonstrated the antinociceptive role of Melatonin in alleviating pain in a rat model of RIFM, potentially induced by glutamatergic dysregulation in the dorsal horn of the spinal cord. The observations regarding Melatonin's antinociceptive effect include (i) reduced motor impairments

with enhanced mechanical and thermal nociception; (ii) phenotypic improvement, indicated by a minimized rat grimace scale; (iii) amendment of histopathological abnormalities in the DRG; (iv) increased expression of MT1 and MT2 receptors, along with a reduction in glutamate neurotransmission, as indicated by decreased levels of PSD95 and synaptophysin in DRG and VGLUT, PSD95, NMDA,

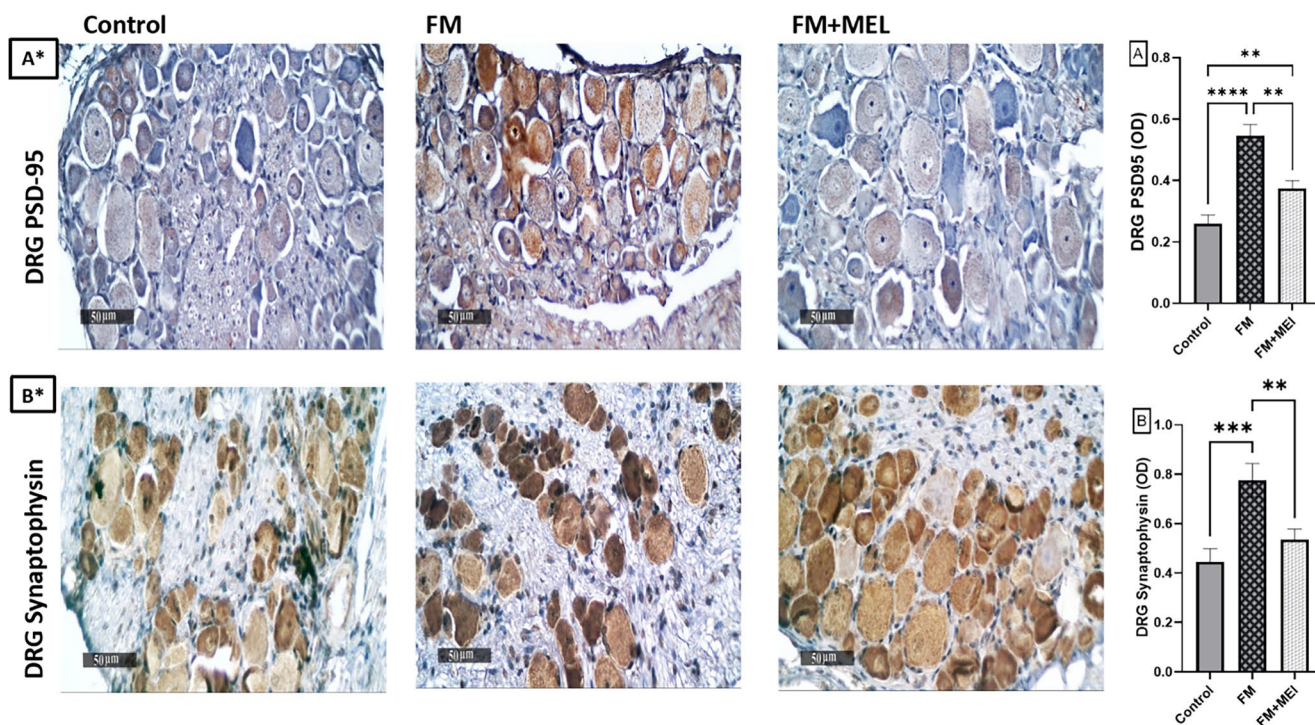


Fig. 8 Impact of Melatonin (10 mg/kg, oral, 3 days) on the immunohistochemically expression of (A* & A) PSD-95 and (B* & B) synaptophysin in the DRG of the FM-like model. Panel (A* & B*) shows an increased number of positively stained brown nuclei (black arrow=positive nuclei), indicating elevated PSD-95 and synaptophysin protein expression in reserpinized rats compared to the unstained DRG tissue in the control group. In contrast, Melatonin treatment

reduced excitotoxicity, as evidenced by fewer brown-stained nuclei (black arrow=positive nuclei). Each bar in panels A and B with a vertical line represents the mean±SD of rats ($n=3$ per group), analyzed using one-way ANOVA followed by Tukey's post-hoc test ($*p<0.05$, $**p<0.01$, $***p<0.001$, and $****p<0.0001$). DRG: dorsal root ganglia, FM: fibromyalgia model, MEL: Melatonin, PSD-95: postsynaptic density protein

and AMAPA levels in spinal cord; (v) mitigation of mitochondrial dysfunction through activation of the SIRT1/PGC-1 α pathway; (vi) alleviation of neuroinflammation, as evidenced by reduced microglial activation, recognized by reduced Iba-1 levels and accompanied by decreased levels of pro-inflammatory factors such as IL-1 β , IL-6, and TNF α ; and (vii) low expression of pro-inflammatory cytokine regulators, including p38 MAPK and NF- κ B. Notably, this study highlights the significance of Melatonin-mediated antinociceptive action in repairing mitochondrial dysfunction, glutamatergic dysregulation, and microglial activation, all of which contribute to the disruption of the ascending pain pathway in the DRG of the FM rat model. Concomitantly in the present study, RIFM rats exhibited heightened pain sensitivity, neuromuscular disability, and motor incoordination, as indicated by their performance in the neurobehavioral assessments.

Reserpine significantly decreased pain threshold in the von Frey test, Randall-Sellito test, cold allodynia, and hot plate tests. Additionally, supraspinal pain was increased, as noted in the tail suspension test. These outcomes are consistent with previous research findings (Brum et al. 2020, 2022). De la Luz-Cuellar et al. (2019) demonstrated that

mechanical allodynia and muscle hyperalgesia peak on the 7th day following Reserpine administration, with symptoms gradually subsiding by day ten (De la Luz-Cuellar et al. 2019). In the present study, Melatonin was started on day 7 and continued for three consecutive days, prompting consideration of whether the observed effects are attributable to Melatonin or influenced by the spontaneous resolution of Reserpine-induced nociception. The current findings revealed that mechanical and thermal hypersensitivity persisted in the Reserpine-only group after day seven, confirming that the pronociceptive effects of Reserpine remained active at the initiation of Melatonin. This persistence strongly supports the conclusion that the attenuation of pain behaviors in the Melatonin-treated group results from Melatonin's pharmacological efficacy rather than spontaneous recovery. Moreover, prior investigations on RIFM have established a prolonged pain state lasting a minimum of 14 days and in some cases extending up to 21 days post-administration (Fusco et al. 2019; Yao et al. 2020; Mohamed et al. 2025; Shafiek et al. 2025a, b; Kamaly et al. 2025). Additionally, Melatonin, and its agonists and antagonists were administered intrathecally before or after intradermal capsaicin injection, where Melatonin and its agonists

significantly decreased mechanical allodynia and hyperalgesia. In contrast, Melatonin antagonism increased the pain withdrawal frequency (Tu et al. 2004). Altogether, these findings revealed that activation of the endogenous Melatonin system in the spinal cord can mitigate central sensitization in FM. These data validate the therapeutic potential of Melatonin in mitigating sustained nociceptive sensitization.

Regarding motor performance in the Rota rod, Reserpine significantly impaired Rota rod performance, simulating motor deficits observed in FM. This finding aligns with previous studies reporting neurobehavioral impairments following Reserpine administration (Yao et al. 2020; Atta et al. 2023b; Mohamed et al. 2025; Kamaly et al. 2025). However, some studies have recorded no significant changes or milder deficits in Rota Rod performance, reflecting differences in experimental protocols, such as training frequency. In the present study, the three training sessions may have pronounced fatigue effects, while another study with fewer training sessions (Zhang et al. 2016) reported less notable deficits. Melatonin demonstrated its potential to improve motor performance in various models, including stroke (Zhao et al. 2024), Parkinson's disease (Rasheed et al. 2018), peripheral neuropathy (El-Sawaf et al. 2024), and cerebral ischemia-reperfusion injury (Yilmaz et al. 2023), particularly at lower doses not exceeding 20 mg/kg. Some studies indicate that higher doses (120–150 mg/kg) of Melatonin may impair Rota rod performance or show limited efficacy, showing the importance of dose consideration (Arreola-Espino et al. 2007; Çakirgöz et al. 2025). Additionally, delayed effects of Melatonin on motor impairment have been reported in models rather than FM, where motor recovery was noted after 7 days of treatment despite no improvement after 3 days (Yilmaz et al. 2023). These discrepancies may be attributed to differences in pathology, treatment duration, and timing across models.

Abnormal pain sensitization is attributed to excessive glutamatergic neurotransmission in the spinal dorsal horn of FM. Contrary to the normal state, glutamate receptors involved in nociceptive transmission, such as NMDARs, are over activated due to increased glutamate release from dorsal horn terminals, which enhances spinal wind-up and hyperalgesia (Staud and Domingo 2001; Pereira and Goudet 2019). Ferrari et al. (2014) demonstrated that intrathecal administration of glutamate increased the central nociceptive reflex excitability in the DRG. In contrast, intrathecal administration of NMDA receptor antagonists reversed this milieu in experimental chronic pain models (Zhou et al. 2011; Ferrari et al. 2014). Unfortunately, NMDAR antagonists produce inconsistent results or cause severe side effects. This underscores the necessity to develop new therapies targeting glutamatergic dysregulation to alleviate pain hypersensitivity without blocking glutamate receptors (Temmermand et al. 2022).

Consistent with the previously mentioned pain-exacerbating mechanism, this study investigated the glutamatergic alteration in the dorsal horn spinal cord and in the DRG. The present results demonstrated a significant upregulation of VGLUT, PSD95, NMDA, and AMPA receptor expression within the dorsal horn. These alterations indicate enhanced glutamatergic neurotransmission and synaptic strengthening, which are hallmark features of central sensitization. The concurrent increase in both presynaptic VGLUT and postsynaptic PSD95, NMDA, and AMPA markers suggests an overall facilitation of excitatory synaptic drive, further supporting the transition of pain. Similar observations have been reported in various chronic pain models, where spinal glutamatergic overactivity plays a critical role in maintaining persistent pain states (Liu and Salter 2010; Niciu et al. 2012; Bardoni 2013; Turan Yücel et al. 2023; Jang and Garraway 2024).

The synaptic scaffolding molecule, PSD-95, binds to the NMDA 2B subunit (NR2B). The binding of NR2B to PSD-95 contributes to dorsal horn neuron hyperexcitability and elevated pain-associated behaviors, such as hyperalgesia and allodynia, in human and animal models. Subsequently, NR2B activation is involved in promoting central sensitization and nociplastic pain (d'Mello et al. 2011; Li et al. 2022). In addition to the dorsal horn, PSD-95 upregulation in the DRG further supports the occurrence of widespread synaptic plasticity contributing to the maintenance of chronic pain. The present model corroborates previous findings of synapse-like remodeling around DRG somata in chronic pain and synaptic marker enrichment in human DRG neurons revealed through increased PSD95 and synaptophysin levels in the DRG (Sun et al. 2006; Cheng et al. 2015; Yu et al. 2024). The expression levels of PSD-95 and synaptophysin in rat DRG as each is linked to glutamatergic neurotransmission in the nervous system. The current study showed a remarkable increase in synaptophysin levels in the DRG after RES injection in rats. These findings support that the dorsal horn of the spinal cord and the DRG glutamatergic system regulate hyperalgesia in FM.

Melatonin demonstrated neuroprotective, anti-inflammatory, and anti-apoptotic efficacy. Herein, the novelty lies in revealing Melatonin's potential to counteract excessive glutamatergic dysregulation and the nociplastic state in FM. This was confirmed by immunohistological analysis, which showed that DRG tissues in the Melatonin-treated group exhibited lower PSD-95 and synaptophysin expression, as well as decreased NR2B expression in the spinal cord. This reflects Melatonin's ability to amend the exaggerated glutamatergic transmission observed in FM, thereby alleviating pain transmission, as seen in behavioral tests. These findings align with a previous clinical study on Melatonin's impact on the pain modulatory system in female FM

patients. Which demonstrated that Melatonin enhanced pain reduction, as reflected by improvements in the outcomes of the visual analog scale, heat and pressure pain thresholds, FM impact questionnaire, and numerical rating pain scale (de Zanette et al. 2014).

Additionally, preclinical studies indicated that the injection of Melatonin significantly suppresses or completely eliminates wind-up activity in the spinal cord of rats. This suppressive action is likely associated with Melatonin's agonistic properties at its receptors in dorsal horn neurons (Laurido et al. 2002; Nosedá et al. 2004). However, the molecular mechanism by which Melatonin exerts its neuroprotective action remains unclear. MT1 and MT2 are two distinct families of membrane receptors located in the plasma membrane, both of which are abundantly expressed in the CNS. Both receptors have been identified in the dorsal horn of the spinal cord, particularly in laminae I-V and X, which are involved in pain regulation mechanisms (Srinivasan et al. 2012; Das et al. 2013). The present research emphasizes their role in the FM experimental model, as their expression levels were inversely proportional to glutamate and pain hypersensitivity. This is consistent with Das et al. (2013); the study confirmed the contribution of MT1/2 in protecting neurons from glutamatergic dysregulation (Das et al. 2013). The knockdown of endogenous MEL receptors was performed using small interfering RNA to elucidate the receptor-dependent neuroprotective efficacy of Melatonin. Results from the silenced endogenous MT1 and MT2 receptors support the function of Melatonin receptors in modulating cellular responses to excitotoxic injury (Das et al. 2013).

The apparent discrepancy between Melatonin's agonist activity and the observed upregulation of MT1 and MT2 mRNA in the present study can be explained by the context-dependent regulatory dynamics of Melatonin receptors in pathological states. In neurodegenerative amyotrophic lateral sclerosis and Huntington's disease, as well as age-related pathologies, MT1/MT2 receptors are frequently downregulated due to oxidative stress and mitochondrial dysfunction. Melatonin prevented the disease-associated decline in MT1 protein in spinal motor neurons, as confirmed by immunostaining and mRNA analyses (Wang et al. 2011; Zhang et al. 2013; Jenwitheesuk et al. 2017; Romano et al. 2024). In the context of FM, a condition linked to oxidative stress and mitochondrial dysfunction, the observed mRNA was downregulated in the disease and reversed by Melatonin administration. This restoration may be owed to re-establishing homeostatic receptor expression in pathological statuses.

Besides glutamatergic dysregulation, mitochondrial dysfunction and neuroinflammation were investigated as potential causes of FM. Maintaining mitochondrial function is critical for managing sensory and chronic pain

(Tu et al. 2004; Flatters 2015). However, few existing studies provide insight into the relationship between Melatonin receptors and mitochondrial biogenesis in DRG. The results revealed that Melatonin efficiently alleviated pain hypersensitivity in animal models by boosting SIRT1 and its substrate, PGC-1 α . These results align with in vitro data suggesting that paclitaxel disrupts mitochondrial membrane potential and metabolic activity in DRG cells, which are subsequently restored by Melatonin (Galley et al. 2017). Melatonin can traverse cell membranes and accumulate inside mitochondria, interacting with mitochondrial MT1 receptors to regulate mitochondrial function. Moreover, Melatonin preferentially accumulates in mitochondria and counteracts mitochondrial-derived ROS by binding to MT1 and MT2, resulting in analgesic effects in several neuropathic pain models (Zeng et al. 2023).

Recent research found that the aberration of the SIRT1/PGC-1 α signaling pathway is involved in the development of neuropathic pain. SIRT1 knockdown in naïve rats resulted in pain behavior (Ling-Jun Xu et al. 2023), while intrathecal administration of the SIRT1 activator SRT1720 drastically decreased chronic constriction injury-induced allodynia (Lv et al. 2015). Notably, SIRT1/PGC-1 α levels were significantly decreased in the spinal cord of a rat model, causing mitochondrial dysfunction and overproduction of pro-inflammatory cytokines, including IL-1 β and TNF- α , which sensitize neurons and induce neuropathic pain (Zeng et al. 2023). Furthermore, SIRT1-mediated deacetylation of PGC-1 α mitigated glutamatergic dysregulation in cortical neurons (Jia et al. 2016). Zeng et al. (2023) highlighted Melatonin's ability, via MT2, to improve mitochondrial function and mitigate neuropathic pain through the SIRT1/PGC-1 α pathway in the DRG of rats (Zeng et al. 2023). Similarly, the present study demonstrated that Melatonin injection promotes SIRT1, which activates PGC-1 α through deacetylation. Previously, Melatonin showed comparable therapeutic effects against FM by targeting key pathological mechanisms, including oxidative stress, inflammation, mitochondrial dysfunction in muscle tissue, and neuroimmune activation in the brain. It has been shown to alleviate motor impairments and improve musculoskeletal structure by reducing inflammation, mitochondrial dysfunction, and oxidative stress markers in the gastrocnemius muscle, indicating its potential in managing FM-related musculoskeletal damage (Favero et al. 2017, 2019). This explains part of the antinociceptive effect of Melatonin, particularly its role in restoring mitochondrial function and alleviating glutamatergic dysregulation, as investigated in RIFM.

Interestingly, earlier research indicated that microglial activation in the spinal cord contributes to neuroinflammation and chronic pain conditions like FM by influencing glutamate release. This process involves the activation of

microglia to the M1 phenotype in the dorsal horn, triggering the release of pro-inflammatory cytokines, including TNF α and IL-1 β /-6, as well as glutamate, leading to neuroinflammation, synaptic hyperexcitability, and central sensitization. The present study examined microglial status in the spinal cord, where Iba1 immunoreactivity was markedly elevated in activated microglia, consistent with previous findings (Ito et al. 1998; Jung et al. 2020). Conversely, Melatonin significantly reduced Iba1 expression levels, potentially through the NF- κ B and MAPK pathways, which have been identified as key regulators of central sensitization besides the role of pro-inflammatory cytokines, IL-1 β , and TNF- α (Fusco et al. 2019). This was evident from inhibiting the p38/MAPK pathway, which restricted microglial activation and neuroinflammation, subsequently alleviating allodynia and hyperalgesia (Bennett 2005). The current results and those of Bennett (2005) reinforce the potential of p38 MAPK pathway inhibitors as a novel approach to pain management in FM patients (Bennett 2005). In the present study, Melatonin-treated rats exhibited downregulation of NF- κ B and p38 MAPK, which may be partially attributed to mitochondrial restoration. PGC-1 α activation has been demonstrated to inhibit NF- κ B signaling in the brain, thereby preventing the release of pro-inflammatory molecules such as TNF α . This promotes microglial activation, with further research needed to elucidate its activation (Yang et al. 2017; Qin et al. 2018; Castro et al. 2022), as is well observed here in Melatonin-treated animals.

The primary limitation of this study is the lack of investigation into the distinct roles of MT1 and MT2 receptors in modulating neuroinflammation and pain signaling within the spinal cord and DRG in the FM animal model. Additionally, the effects of Melatonin on descending pain modulatory pathways, including serotonergic and noradrenergic neurotransmission, were not explored. Body weights were also not monitored throughout the entire experimental period, and assessing Iba1 alone did not clarify the microglial phenotypes. Addressing these gaps is essential for a comprehensive understanding of receptor-specific mechanisms underlying Melatonin's analgesic and anti-inflammatory effects.

Compliance with ethical standards

PT 3478 is the approval number assigned to this investigation by the Animal Care and Use Ethics Committee of the Faculty of Pharmacy, Cairo University. The study was conducted in accordance with the ARRIVE 2020 standards and the guidelines specified in the Guide for the Care and Use of Laboratory Animals, which was published by the US National Institutes of Health (NIH publication No. 85-23,

revised 2011). The study's animals were subjected to all feasible safeguards to mitigate their distress.

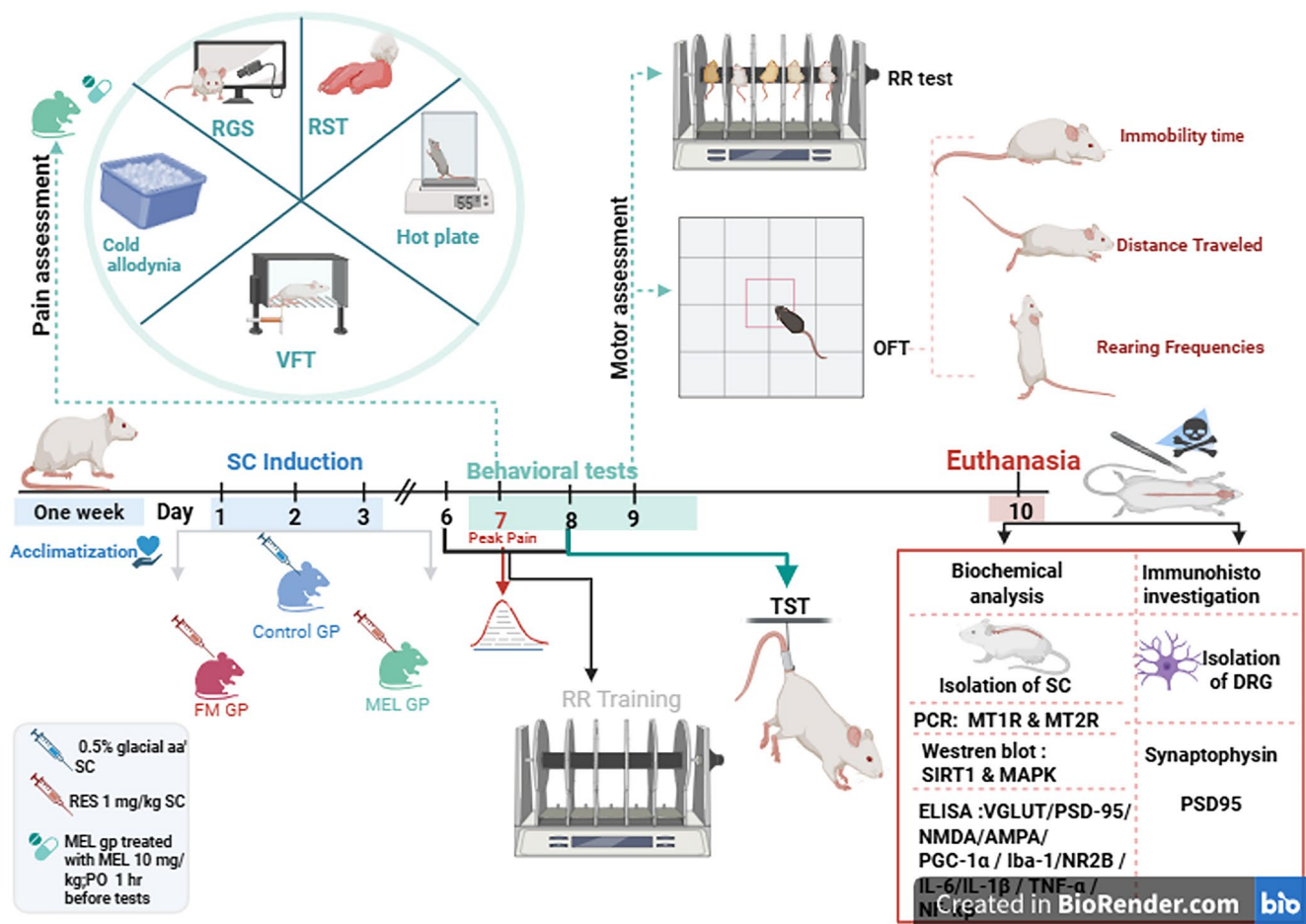
Induction of the FM-like Model

In the neck region, RES (Sigma-Aldrich, Saint Louis, MO, USA) was administered subcutaneously (s.c.) at a dose of 1 mg/kg/day for three days. The preparation was a 0.5% glacial acetic acid solution (Nagakura et al. 2009).

Experimental Design

In Scheme 1, the animals were randomly assigned to three groups, each consisting of ten rats. In order to dispel allocation bias, a random number generator was implemented. With a power of 0.8, an effect size of 0.6, and an alpha level of 0.05, the G*Power calculator version 3.1 (Düsseldorf, Germany) was employed to determine the sample size.

Group I served as the control group, receiving distilled water with 0.5% glacial acetic acid (s.c.) for three days, followed by distilled water with 1% DMSO. Group II, designated as the FM group, received RES (1 mg/kg, s.c.; Sigma-Aldrich, MO, USA) for three days. Group III (FM+Melatonin) received RES (1 mg/kg, s.c.) for three days, followed by Melatonin (10 mg/kg, p.o.; Sigma-Aldrich, MO, USA) dissolved in 1% DMSO in distilled water, administered for three days starting on day 7, one hour before behavioral testing (Galley et al. 2017). Melatonin's dose was selected based on extensive preclinical evidence demonstrating its efficacy in alleviating pain without inducing motor or sedative side effects (Galley et al. 2017; Tancheva et al. 2021). On the seventh day, the pain reached its peak, consistent with previous findings (De la Luz-Cuelar et al. 2019; Ikeda et al. 2023). The Grimace scale was used to assess spontaneous pain, while mechanical and thermal sensitivities were evaluated using Von Frey, Randall-Sellito, hind paw cold allodynia, and hot plate tests. The next day, the TST was performed to assess supraspinal pain. On the ninth day, behavioral tests examining motor activity, muscle fatigue, and coordination were conducted using the open field (OFT) and rotarod (RR) tests. Motor behavior was subsequently recorded using the ANY-maze video tracking software (Stoelting Co., Illinois, USA). Researchers were blinded to group assignments during behavioral assessments and data analysis to minimize observer bias. A two-hour resting period was implemented between tests, beginning with the least stressful and concluding with the most stressful, to mitigate animal suffering. The testing sequence was chosen to avoid prior mild thermal stimulation that can sensitize animals to subsequent mechanical stimuli (Gröne et al. 2012). All instruments were sanitized



Scheme 1 Experimental study diagram. AMPA receptor: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; DRG: dorsal root ganglia; ELISA: enzyme-linked immunosorbent assay; FM GP: fibromyalgia group; Iba-1: binding adaptor molecule 1; IL-1 β : interleukin-1 beta; IL-6: interleukin-6; MAPK: mitogen-activated protein kinases; MEL GP: Melatonin group; MT1R: Melatonin receptor 1; MT2R: Melatonin receptor 2; NF- κ B: nuclear factor kappa B; NMDA: N-methyl-D-aspartate receptors; NR2B: NMDA receptor subunit 2B;

OFT: open field test; PCR: polymerase chain reaction; PGC-1 α : peroxisome proliferator-activated receptor gamma coactivator 1-alpha; p.o.: per oral; PSD95: postsynaptic density protein 95; RES: Reserpine; RGS: rat grimace scale; RR: rotarod; RST: Randall-Selitto mechanical threshold; s.c.: subcutaneous; SIRT1: silent information regulator sirtuin 1; TNF- α : tumor necrosis factor-alpha; TST: tail suspension test; VGLUT: vesicular glutamate transporter; VFT: von Frey test

with 70% ethanol after each experiment. Behavioral evaluations were conducted according to the established norms and standard practices of the Neurobehavioral Core Lab (Faculty of Pharmacy, Cairo University). All data collectors and outcome assessors remained blinded to treatment groups until data analysis was completed.

Tissue Collection

Animals were euthanized through decapitation under pentobarbital anesthetic (200 mg/kg, i.p.) after the completion of all behavioral evaluations (Zatroch et al. 2016). The spinal cords were individually isolated, cleansed with saline, and weighed. Spinal cords were divided into two subgroups. The first subgroup ($n=3$) was preserved in 10% neutral buffered formalin and utilized for histological and

immunohistochemical analysis of DRG. The second subgroup from the remaining rats' spinal cords were excised, swiftly frozen in liquid nitrogen, and stored at -80°C . To ensure consistent analysis, each spinal cord was divided rostro-caudally into two equal halves (upper and lower segments). The lower half of the study ($n=6$) was utilized to VGLUT, PSD95, NMDA, AMPA, PGC1 α , NF- κ B, Iba-1, TNF- α , IL-6/ -1β , and NR2B using the ELISA technique. The upper half of the samples was used to evaluate MT1R and MT2R via quantitative real-time polymerase chain reaction (qPCR) ($n=3$), and SIRT-1 and MAPK were assessed using western blot ($n=3$). The remaining spinal cord tissue was stored at -80°C for possible further validation analyses. In Scheme 1, the experimental design is illustrated.

isoxazolepropionic acid receptor; DRG: dorsal root ganglia; ELISA: enzyme-linked immunosorbent assay; FM

GP: fibromyalgia group; Iba-1: binding adaptor molecule 1; IL-1 β : interleukin-1 beta; IL-6: interleukin-6; MAPK: mitogen-activated protein kinases; MEL GP: Melatonin group; MT1R: Melatonin receptor 1; MT2R: Melatonin receptor 2; NF- κ B: nuclear factor kappa B; NMDA: N-methyl-D-aspartate receptors; NR2B: NMDA receptor subunit 2B; OFT: open field test; PCR: polymerase chain reaction; PGC-1 α : peroxisome proliferator-activated receptor gamma coactivator 1-alpha; p.o.: per oral; PSD95: postsynaptic density protein 95; RES: Reserpine; RGS: rat grimace scale; RR: rotarod; RST: Randall-Selitto mechanical threshold; s.c.: subcutaneous; SIRT1: silent information regulator sirtuin 1; TNF- α : tumor necrosis factor-alpha; TST: tail suspension test; VGLUT: vesicular glutamate transporter; VFT: von Frey test.

Neurobehavioral Tests

Rat Grimace Scale

A video camera was used to assess spontaneous pain in rats by capturing images of their facial expressions every 3 min, resulting in 10 photographs over 30 min. The photographs were then randomly organized in PowerPoint for evaluation on a 3-point scale. They were assessed based on four distinct action units: orbital tightness (eye), ear alteration, nose/cheek flattening, and whisker modification. Each action unit was scored on a 3-point scale (0=nonexistent, 1=moderately visible, 2=definitely present) (Nagakura et al. 2019; Tanei et al. 2020).

Open Field Test (OFT)

The open field test (OFT) was employed to evaluate the spontaneous locomotor activity of animals. A black box measuring 80 cm by 80 cm and 40 cm in height was used for this test. Each rat was positioned in the center of the box and allowed to wander autonomously for three minutes. The behavior of the rat was captured by a video camera that was positioned above the box. The camera recorded the total distance traveled in meters (OFT DT), immobility time (the extent of lack of movement during testing, OFT IT), and the frequency of horizontal locomotor and vertical activity, known as rearing (OFT RF). Cleaning the apparatus with 70% ethanol following each animal trial and allowing it to dry entirely before the next trial was conducted to prevent any potential bias from aroma (Ibrahim et al. 2024).

Von Frey Test (VFT)

The VFT uses specialized filaments to determine the mechanical allodynia thresholds in rats. A manual protocol

was employed to assess the mechanical sensitivity of the animals. After a 30-minute acclimatization period on a raised mesh floor in an acrylic cage, the rats' hind legs were stimulated five times using calibrated von-Frey filaments. The minimal force-inducing withdrawal response was established as the mechanical threshold, and a positive response was recorded. A response was considered positive if the animal displayed any nociceptive activity, such as rapid paw withdrawal, licking, or shaking of the paw, either during stimulus administration or immediately following filament removal, and was documented as paw withdrawal threshold (Deuis et al. 2017; Ikeda et al. 2023).

Rotarod (RR) Test

The RR was deployed to assess fatigue-like symptoms in rats administered RES to induce FM. Before the experimental procedures, the rats underwent training for three consecutive days, with each session lasting one minute, on an automated five-lane RR device (Model 47750, Ugo Basile, Italy) that accelerated from 4 to 20 rpm. On the fourth day, a fatigue analysis was conducted at 20 rpm, and the time the rats remained on the rod was documented as rotarod falling latency (RR FL) (Dagnino et al. 2019).

Randall-Selitto Test (RST)

To assess mechanical hyperalgesia, the RST was implemented. The analgesiometer (Model 7200, Ugo Basile, Italy) was employed to evaluate the withdrawal threshold by applying a continuous mechanical force to the mid-gastrocnemius muscle of the rat's rear limb. The Randall-Selitto mechanical threshold (RS MT) was determined by recording the hind limb withdrawal of rats under restraint with a soft cotton fabric. In an effort to guard against tissue injury, the threshold burden was restricted to 250 g (Kamaly et al. 2025).

Hind Paw Cold Allodynia Test

The hind paw cold allodynia test is employed to assess cold thermal sensitivity by assessing the withdrawal response of the rat's hind paw when it is delicately immersed in ice-cold water at 4 ± 1 °C. We assessed cold allodynia using paw withdrawal latency (CAPWL), with a threshold duration of 20 s (Ibrahim et al. 2024).

Hot Plate Test

The hot plate test is a commonly used technique for evaluating supraspinal thermal nociception in rats and is often employed to detect thermal hyperalgesia. In this experiment,

rats are positioned on a confined hot plate maintained at a temperature of 55 ± 1 °C, and the time taken to lick a hind paw or escape the enclosure is designated as hot plate reaction latency (HP RL), indicating the threshold of thermal pain response in rats. A cutoff reaction time of 20 s is used to prevent physical injury (Ibrahim et al. 2024; Kamaly et al. 2025).

Tail-suspension Test (TST)

The tail suspension test is based on the fact that a rat, when hung by its tail, displays alternating phases of agitation and stillness. In this experiment, animals were elevated 50 cm off the ground using adhesive tape, with their forelimbs stabilized by two smooth, V-shaped slopes to prevent excessive agitation. The duration of immobility was quantified during a 6-minute trial and recorded as the tail suspension test immobility time (TST IM) (Ibrahim et al. 2024).

Histopathological Examination

Rat dorsal root tissue samples from lumbar segments were preserved in 10% neutral buffered formalin for 72 h and subsequently subjected to a series of ethanol grades, cleaned with xylene, and then infiltrated and embedded in Paraplast tissue embedding medium (Leica Biosystems). Serial slices of dorsal root ganglion tissue, each five micrometers thick, were prepared using a rotary microtome for demonstration. Tissue slices were stained with hematoxylin and eosin (H&E) using the standard staining process for blinded light microscopic inspection conducted by an expert histologist. Culling (2013) states that all normal protocols for sample processing were followed (Culling 2013).

Immunohistochemical Examination

In accordance with the manufacturer's protocol, immunohistochemistry staining was performed on paraffin-embedded DRG tissue slices that were 5 microns thick. 0.3% H₂O₂ was applied to deparaffinized dorsal root ganglion tissue segments for a duration of 20 min. The DRG was incubated at 4 °C overnight with an anti-PSD95 antibody (Abcam, ab192757, 1:200) and an anti-synaptophysin antibody (Abcam, ab14692, 1:100). The HRP Envision kit secondary antibody (Cat.#K5007) was incubated with the tissue sections for 20 min after they were rinsed with PBS. The specimens were subsequently cleansed and subjected to diaminobenzidine (DAB) treatment for 15 min. Subsequently, they were washed with PBS, counterstained with hematoxylin, dehydrated, cleared in xylene, and mounted for microscopic analysis. The mean PSD95 and synaptophysin optical densities in immunohistochemically stained

sections were determined by randomly selecting and examining a minimum of six non-overlapping fields. The mean of these 6 values was averaged for each animal, and then statistical analyses were performed on these per-animal means as independent data points. The histologist utilized the Leica application module for histological analysis, which was integrated with a Full HD microscopic imaging system (Leica Microsystems GmbH, Germany) (Culling 2013).

Biochemical Parameters

Enzyme-linked Immunosorbent Assay (ELISA)

Rat-specific ELISA kits were deployed to ascertain the following parameters in the tissue samples of the SC: PGC1 α (Cat.#MBS1600213), Iba-1 (Cat.#MBS3809193), NF- κ B (Cat.#MBS2505513), and IL-1 β (Cat.#MBS825017; all from My BioSource, San Diego, CA, USA). Cusabio ELISA kits were used to evaluate TNF- α (Cat.#CSB-E11987r, Houston, TX, USA). In addition, ELISA kits from R&D Systems were used to quantify IL-6 (Cat.#R6000B, Minneapolis, MN, USA). Moreover, N-methyl-D-aspartate receptor subunit 2B (NR2B) (AFG Bioscience, Cat. No. EK721705, Northbrook, IL, USA), VGLUT (My BioSource, San Diego, CA, USA; Cat.#MBS2611774), PSD95 (Sandwich ELISA; LS-F7142, LSBio, Seattle, WA, USA), NMDA (My BioSource, San Diego, CA, USA; Cat.#MBS269995), and AMPA (ELK Biotechnology, Cat.# ELK6368, Wuhan, Hubei, China) were assessed according to the manufacturer's instructions. AMPA, TNF- α , NF- κ B, and IL-6/IL-1 β were quantified in pg/mg protein, while VGLUT, PSD95, NMDA, PGC1 α and NR2B were measured in ng/mg protein. Protein concentrations were assessed using a BCA kit from G-Bioscience (USA), employing a standard curve to convert measurements to pg/ml or ng/ml. These values were normalized by dividing by the tissue protein concentration (mg/ml), yielding expressions in pg/mg or ng/mg protein.

Western Blot of P38 MAPK and SIRT1

Following the quantification of protein levels with the BCA test kit (Bio-Rad, CA, USA), the spinal cord's SIRT1 and MAPK protein expressions were evaluated. Using SDS-PAGE, equal portions of protein samples were separated and subsequently deposited onto a nitrocellulose membrane. Subsequently, the membranes were incubated at 4 °C overnight with primary antibodies that were specific to SIRT1 (Cat.#PA5-116530) and phospho-p38 MAPK (Cat.#36-8500), sourced from Thermo Fisher Scientific, MA, USA. Later, an appropriate horseradish peroxidase-conjugated secondary antibody (Dako, Glostrup, Denmark) was applied and incubated overnight. Following this, the bands were visualized using the Western Lightning

Table 1 Primer sequences

| Parameters | Primer sequences |
|------------|--|
| MT1R | F:5-CGGACAGCAAACCCAACTG-3 R:5-AACTAGCCACGAAGAGCCAC-3 |
| MT2R | F:5-CATCTGTCACAGTGCACCT-3 R:5-TGCTGGCTGTCTGGATGAAG-3 |
| B-actin | F:5-CCCGCGAGTACAACCTTCTT-3 R:5-AACACAGCCTGGATGGCTAC-3 |

Plus ECL chemiluminescence reagent (Perkin Elmer, MA, USA), which was captured using a Chemi-Doc imager (Bio-Rad, CA, USA). The optical density of the findings was standardized to β -actin (Cat.#PA1-183) for SIRT1 and total phospho-p38 MAPK (Cat.#AHO1202), both from Thermo Fisher Scientific, MA, USA.

qPCR Experiment

Utilizing the $2^{-\Delta\Delta CT}$ equation, the relative expression of spinal MT1R and MT2R mRNA in relation to β -actin was determined. Utilizing the SV total RNA isolation system (Cat.#Z3101; Promega, WI, USA), total RNA was extracted and subsequently transformed to cDNA using an Invitrogen kit (CA, USA). SYBR Green PCR master mix (Cat.#4309155; Applied Biosystems, CA, USA) was employed to run the qPCR. Table 1 contains primer sequences for the parameters that were examined.

Statistical analysis

Mean \pm standard deviation was used for representing the data, and significance was established at $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$. The means were compared using a one-way analysis of variance (ANOVA) and Tukey's multiple-comparison test in Graph-Pad Prism (version 9; San Diego, CA, USA). Statistics were conducted to analyze the data. The effect size, partial eta squared (η^2), statistical significance (p), degrees of freedom (df), and F-value (F) were specified for each effect.

Conclusion

In conclusion, this study demonstrates that Melatonin exerts significant antinociceptive and anti-inflammatory effects in a Reserpine-induced FM model. Melatonin effectively alleviates nociplastic pain and associated behavioral disturbances by modulating glutamatergic-related synaptic markers in the DRG, restoring mitochondrial function, and reducing microglial activation in the spinal cord. These findings support the potential therapeutic role of Melatonin in managing FM symptoms and highlight its capacity to target molecular abnormalities in pain pathways.

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Author Contributions Ahmed S. Kamel and Jehad Osama: Experimental study, biochemical and histopathological analysis, data interpretation, writing, reviewing, and approval of the manuscript. Nesrine S. EL-Sayed: Approval and supervision of the manuscript. Ghada M Ragab: Writing, supervision, and approval of the manuscript. Amira A. El-Gazar: Writing and supervision of the manuscript.

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Data Availability All relevant data are present in the submitted manuscript, table, and figures. Any other data will be made available on request.

Declarations

Ethics Approval 3478 is the approval number assigned to this investigation by the Animal Care and Use Ethics Committee of the Faculty of Pharmacy, Cairo University. The study was conducted in accordance with the ARRIVE 2020 standards and the guidelines specified in the Guide for the Care and Use of Laboratory Animals, which was published by the US National Institutes of Health (NIH publication No. 85-23, revised 2011). The study's animals were subjected to all feasible safeguards to mitigate their distress.

Competing interests The authors declare no competing interests.

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