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Melatonin and Exercise Restore Myogenesis and Mitochondrial Dynamics Deficits Associated With Sarcopenia in iMS-*Bmal1*$^{-/-}$ Mice

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ABSTRACT

Sarcopenia, a condition associated with aging, involves progressive loss of muscle mass, strength, and function, leading to impaired mobility, health, and increased mortality. The underlying mechanisms remain unclear, which limits the development of effective therapeutic interventions. Emerging evidence implicates chronodisruption as a key contributor to sarcopenia, emphasizing the role of *Bmal1*, a circadian clock gene critical for muscle integrity and mitochondrial function. In a skeletal muscle-specific and inducible *Bmal1* knockout model (iMS-*Bmal1*$^{-/-}$), we observed hallmark features of sarcopenia, including disrupted rhythms, impaired muscle function, and mitochondrial dysfunction. Exercise and melatonin treatment reversed these deficits independently of *Bmal1*. Building on these findings, the present study elucidates several mechanisms underlying these changes and the pathways by which melatonin and exercise exert their beneficial effects. Our findings indicate that iMS-*Bmal1*$^{-/-}$ mice exhibit reduced expression of satellite cell and muscle regulatory factors, indicating impaired muscle regeneration. While mitochondrial respiration remained unchanged, notable alterations in mitochondrial dynamics disrupted mitochondria in skeletal muscle. In addition, these mice showed alterations in muscle energy metabolism, compromised antioxidant defense, and inflammatory response. Remarkably, exercise and/or melatonin successfully mitigated these deficits, restoring muscle health in *Bmal1*-deficient mice. These findings position exercise and melatonin as promising therapeutic candidates for combating sarcopenia and emphasize the need to elucidate the molecular pathways underlying their protective effects.

Yolanda Ramírez-Casas and José Fernández-Martínez contributed equally to this study.

1 | Introduction

As the population ages, sarcopenia emerges significantly, affecting the elderly's functional capacity, mobility, and overall health, while also increasing mortality risk [1]. This condition represents a considerable social concern, impacting not only on individuals' health but also on substantial healthcare expenditures. Despite its clinical relevance, there are currently no fully effective treatments to prevent or reverse muscle wasting during aging, due to an incomplete understanding of its pathophysiological mechanisms. Several factors, including reduced muscle fiber size and number, altered protein synthesis, decreased satellite cell (MuSC) number, and their proliferative and regenerative capacity, as well as inflammation, mitochondrial dysfunction, and oxidative stress, are related to sarcopenia [2, 3]. Moreover, there is growing evidence supporting the link between chronodisruption and aging, as well as its association with the development of many chronic diseases, including sarcopenia [4, 5]. This emphasizes the importance of studying clock genes, particularly *Bmal1*, an activator of the core of the biological clock expressed in skeletal muscle [6, 7]. *Bmal1* links the circadian clock with the immune system [8], plays a crucial role in maintaining skeletal muscle integrity to ensure contractile function [9, 10], and is also involved in preserving muscle mass during aging [11]. Additionally, *Bmal1* contributes to muscle regeneration through pro-myogenic responses and enhances mitochondrial function within cells [12, 13]. As *Bmal1* levels decline with age [14], disruption of the peripheral muscle clock may be associated with sarcopenia.

Besides the benefits of exercise and increased protein intake [15], melatonin (aMT) provides multiple benefits for age-related conditions [16–19] and improves sarcopenia by enhancing muscle function and structure, as well as counteracting mitochondrial damage associated with aging [20–22]. Apart from its chronobiotic properties, this molecule exhibits antioxidative and anti-inflammatory properties and maintains mitochondrial homeostasis by preserving its function, bioenergetics, and dynamics [23–25]. These regulation mechanisms are particularly important in the skeletal muscle, where mitochondria are essential organelles responsible for regulating the metabolic status of this tissue [26].

For deepening the *Bmal1*-sarcopenia connection and the protective effects of exercise and/or melatonin, we used here a skeletal muscle-specific and inducible *Bmal1* knockout model (iMS-*Bmal1*$^{-/-}$) generated by us. We recently showed that these mice develop sarcopenia and exhibited altered activity/rest rhythms, muscle function, and structure, as well as reduced mitochondrial oxidative capacity and mitochondrial damage. Exercise and melatonin counteracted these changes through a *Bmal1*-independent mechanism [21]. However, the molecular mechanisms underlying these changes, as well as how melatonin and/or exercise exert their beneficial effects, remain unknown. Building on our previous results, this study aimed to analyze muscle myogenesis, mitochondrial respiration and dynamics, inflammation, and metabolic pathways, which are involved in sarcopenia, on the gastrocnemius muscle (GM) of iMS-*Bmal1*$^{-/-}$ mice. The goal of this study was to explore how these mechanisms contribute to sarcopenia and to understand the beneficial effects of melatonin and exercise.

2 | Materials and Methods

2.1 | Generation of the Mouse Model and Experimental Groups

A model of inducible skeletal muscle-specific *Bmal1* knockout mice (iMS-*Bmal1*$^{-/-}$) was established using the method described by other authors [27] and elsewhere updated [21]. Cre LoxP activation was performed using tamoxifen, while control animals were treated with vehicle (Figure 1). The housing conditions, experimental design, groups, and treatments were based on the methodology established by Fernández et al. [21]. The administration of these treatments was carried out in accordance with previous studies that have demonstrated their efficacy [20–22, 28, 29]. Details are available in Supporting Information S1: Supporting Information.

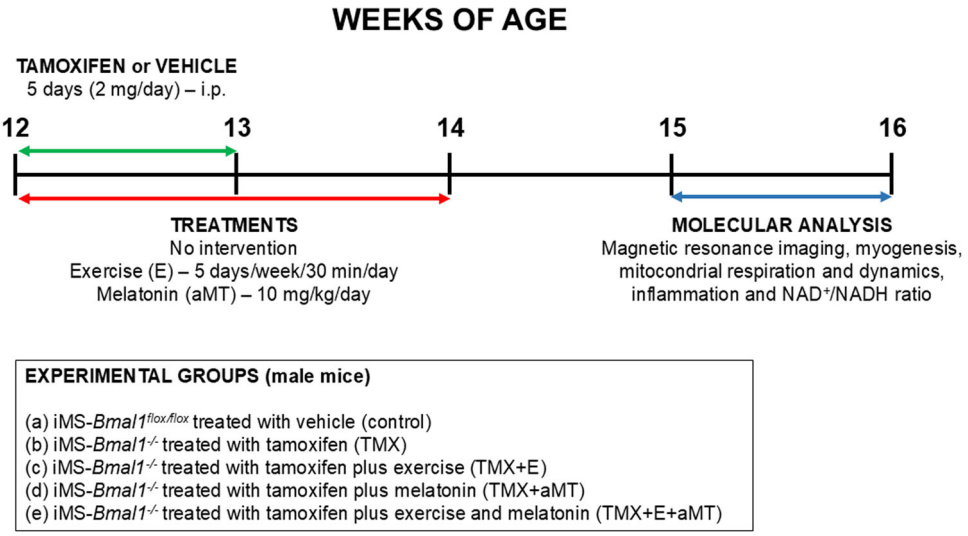

FIGURE 1 | Experimental groups and treatments. Cre LoxP activation with tamoxifen or vehicle was performed at 12 weeks of age. Exercise and/or melatonin treatments were administered for 2 weeks, starting at week 12 of age. Molecular analysis was performed during Week 15 (Figure 1 is adapted from [21]).

Procedures were conducted in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (CETS #123), and the Spanish law for animal experimentation (R.D. 53/2013). This protocol was accepted by the Andalusian's Ethical Committee (#29/05/2020/069).

2.2 | Tissue Preparation

Animals were anesthetized with equithesin (1 mL/kg) and killed at 11:00 am to obtain GMs, ensuring consistency in timing to minimize circadian variations. GMs were stored at −80°C until further analysis. For Magnetic resonance imaging (MRI), the animals were anesthetized with isoflurane (1.5% in air), and freshly dissected GMs were later used for mitochondrial respiration experiments. Details are available in Supporting Information S1: Supporting Information.

2.3 | RNA Extraction and qRT–PCR

RNA extraction was performed on the GM of the different experimental groups using TRIzol RNA Isolation Reagent (15596026, Invitrogen, Thermo Fisher Scientific, Madrid, Spain) according to the manufacturer's instruction. cDNA was then synthesized, normalized to the housekeeping gene β-actin. Details are available in Supporting Information S1: Supporting Information.

2.4 | Magnetic Resonance Imaging (MRI)

The MRI experiments were conducted on anesthetized animals to acquire axial images of the GM, which were subsequently analyzed as previously described [20]. Details are available in Supplementary Information. The results of this study are depicted in the Supporting Information S1: Figure S1.

2.5 | Mitochondrial Respiration

To measure the oxygen consumption rate (OCR), mitochondria were isolated from the GM following the protocol described by Rogers et al. [30]. Mitochondrial respiration was then assessed using an XFe24 Extracellular Flux Analyzer (Agilent, Barcelona, Spain) with sequential respiration states, including proton leak and ATP production. Additionally, rotenone (2 mM) was added to inhibit complex I (CI) during the measurements. Details are available in Supporting Information S1: Supporting Information.

2.6 | Western Blot Experiments

For western blot analysis, GMs were homogenized, and protein concentration was determined by the Bradford method. Proteins were separated by SDS-PAGE electrophoresis, transferred to a PVDF membrane, and incubated with antibodies for target protein quantification. Anti-gapdh was used as a housekeeping protein. Details are available in Supporting Information S1: Supporting Information.

2.7 | Measurement of NAD^+/NADH Ratio

The NAD^+/NADH ratio was measured using a commercial kit following the manufacturer's instructions. NAD^+ and NADH concentrations were quantified using a standard calibration curve and normalized to the protein content, as determined by Bradford assay. Details are available in Supporting Information S1: Supporting Information.

2.8 | Statistical Analysis

Data are expressed as mean ± SEM of $n = 7$ animals per group for RNA extraction and qRT-PCR, western blot analysis experiments, and NAD^+/NADH ratio; $n = 10$ animals per group for mitochondrial respiration (with and without rotenone), of which $n = 5$ animals per group were previously used for MRI. All statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software, Boston, MA, USA). For statistical comparisons, an unpaired *t*-test was used, and significance was taken as $p < 0.05$.

3 | Results

3.1 | iMS-*Bmal1*$^{-/-}$ Mice Exhibit Reduced Myogenesis, Which Is Restored by Exercise and Melatonin Treatment

Given that animals lacking *Bmal1* in skeletal muscle display alterations in muscle morphology and structure that are normalized by exercise and melatonin treatment [21], we investigated here the markers of myogenesis involved in muscle cell proliferation and differentiation. Additionally, we assessed the expression of myostatin (mstn), a myokine associated with muscle mass loss [31, 32]. Specifically, we measured the expression of MuSC (*pax7*), muscle regulatory factors (MRFs) responsible for myofiber differentiation and formation (*myf5*, *myod*, *myog*, and *myf6*), and *mstn* by qRT-PCR (Figure 2). A significant decrease in the expression of *pax7* was detected in the skeletal muscle of animals lacking *Bmal1*, indicating impaired muscle regeneration compared with controls. Treatment with exercise and/or melatonin restored *pax7* expression levels (Figure 2A). Regarding MRFs, a similar pattern was observed, with the expression of *myf5*, *myod*, *myog*, and *myf6* reduced in TMX animals (Figure 2B-E). Both exercise and melatonin exerted protective effects, enhancing *myod*, *myog*, and *myf6* expression and improving myogenesis. However, while exercise and combined treatment significantly recovered *myf5* expression, melatonin alone induced only a mild, non-significant recovery. Finally, the significant decrease in *mstn* expression in *Bmal1*-deficient mice was not restored by the treatments (Figure 2F).

3.2 | iMS-*Bmal1*$^{-/-}$ Mice Exhibit Unaffected Respiration, Which Was Impaired by Exercise and CI Inhibition, With Melatonin Improving Respiration Through CI

Because we previously reported structural alterations in mitochondria with reduced succinate dehydrogenase (SDH)

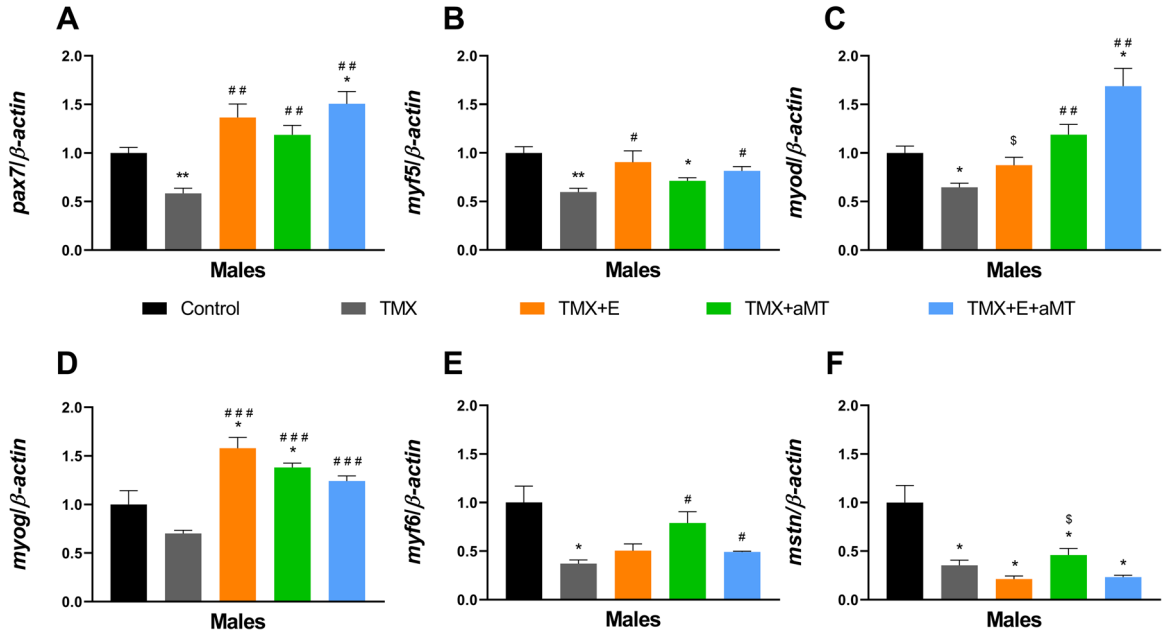

FIGURE 2 | Analysis of the expression of key genes involved in myogenesis, and the protective effects of melatonin and/or exercise in the control, TMX, TMX + E, TMX + aMT, and TMX + E + aMT male mice. (A) Expression of *Pax7* mRNA; (B) expression of *myf5* mRNA; (C) expression of *myod* mRNA; (D) expression of *myog* mRNA; (E) expression of *myf6* mRNA; and (F) expression of *mstn* mRNA. Data are expressed as means ± SEM ($n = 7$ animals/group). $^{*}p < 0.05$, and $^{**}p < 0.01$ versus control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ versus TMX; $^{\$}p < 0.05$ versus TMX + E+aMT.

activity in *Bmal1*-deficient skeletal muscle [21], we sought to explore the molecular underpinnings of these changes in this mouse model. The OCR, a key indicator of mitochondrial oxidative phosphorylation, as well as proton leak and ATP production in isolated mitochondria of GM, were analyzed. Our analysis revealed no significant differences in OCR and respiratory states between control and TMX animals. However, animals undergoing exercise treatment exhibited a notable reduction in these values potentially due to localized mitochondrial damage that we previously revealed [21]. Conversely, melatonin treatment improved respiratory values, although it did not counteract the effects induced by exercise (Figure 3A,C). We also published the reduction in SDH activity in the GM of *Bmal1*-deficient mice [21]. Here, inhibiting CI with rotenone decreased OCR compared with baseline respiration in TMX, possibly indicating an impairment at the complex II (CII) level, and this reduction was also observed across the treatments (Figure 3B,D). Of note, melatonin was unable to enhance respiration in rotenone-treated mitochondria, suggesting that it acts at the CI level (Figure 3D).

Regarding proton leak and ATP production, TMX animals did not exhibit significant deviations from controls. Nonetheless, both exercise and melatonin treatment resulted in a significant decrease in proton leak and an increase in ATP production (Figure 3E). Although OCR values decreased in the TMX + E and TMX + E+aMT groups, the observed improvements in proton leak and ATP production suggest that these treatments compensate for mitochondrial deficiency. Notably, rotenone did not cause significant changes, except in the TMX + E+aMT group, where it resulted in reduced proton leak and increased ATP production (Figure 3F).

3.3 | iMS-*Bmal1*$^{-/-}$ Mice Show Impaired Mitochondrial Dynamics, Which Were Normalized by Exercise and Melatonin Treatment

Alterations in mitochondrial dynamics are linked to the development of sarcopenia, and *Bmal1* is required for maintaining mitochondrial function [33, 34]. We examined here mitochondrial biogenesis, fusion, fission, and mitophagy in the GM of *Bmal1* deficient mice. Regarding mitochondrial biogenesis, we observed a significant decrease in *pgc-1α* mRNA expression in TMX animals compared with controls. Both exercise and melatonin treatment effectively restored its normal expression (Figure 4A). Additionally, the protein levels of Tfam and Nrf1 decreased in TMX animals and even in those treated (Figure 4B,C). These results underscore the critical role of *Bmal1* in maintaining the proper function of these key mitochondrial regulators. Moreover, TMX induced a significant increase in Opa1 compared with the controls, whereas exercise and, especially melatonin, reduced excessive fusion (Figure 4D). The expression of *drp1* decreased in the absence of *Bmal1*, being restored by exercise and/or melatonin treatment (Figure 4E). Conversely, Fis1 levels did not differ significantly between the TMX and control groups; treatments, however, consistently reduced Fis1 levels in all experimental groups (Figure 4F). The protein

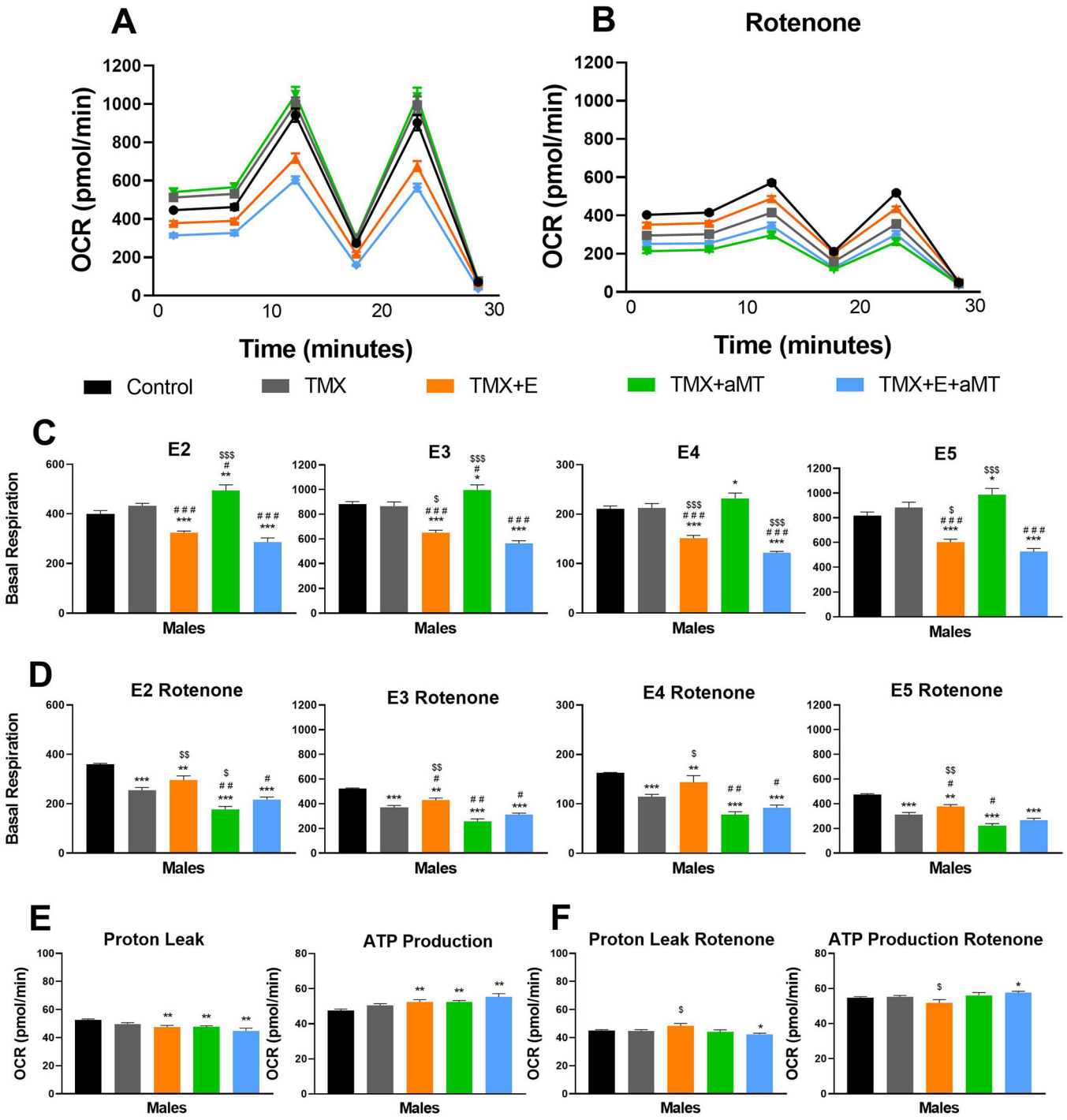

FIGURE 3 | Different respiratory parameters in isolated mitochondria from the GM of iMS-*Bmal1*$^{-/-}$ mice and their controls. (A) Measurement of OCR values in experimental groups (pmol/min); (B) measurement of OCR values in experimental groups using rotenone (pmol/min); (C) respiratory state values in experimental groups (pmol/min); (D) respiratory state values in experimental groups using rotenone (pmol/min); (E) proton leak and ATP production measurements in experimental groups; and (F) proton leak and ATP production measurements in experimental groups using rotenone. Data are expressed as means ± SEM ($n = 10$ animals/group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus TMX. $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ versus TMX + E+aMT.

levels of Bnip3, Pink1, and Parkin were analyzed for mitophagy. Both Bnip3 and Parkin levels decreased significantly in TMX animals, but they did not recover after treatments (Figure 4G,I). In contrast, Pink1 levels remained unchanged in mice with *Bmal1* loss in skeletal muscle (Figure 4H).

3.4 | iMS-*Bmal1*$^{-/-}$ Mice Exhibit Changes in the NAMPT-NAD^{+}-SIRTs Axis

The circadian clock gene *Bmal1* enhances nicotinamide phosphoribosyl transferase (NAMPT) expression, boosting NAD^{+} synthesis and activating Sirt1 and Sirt3, while rhythmically

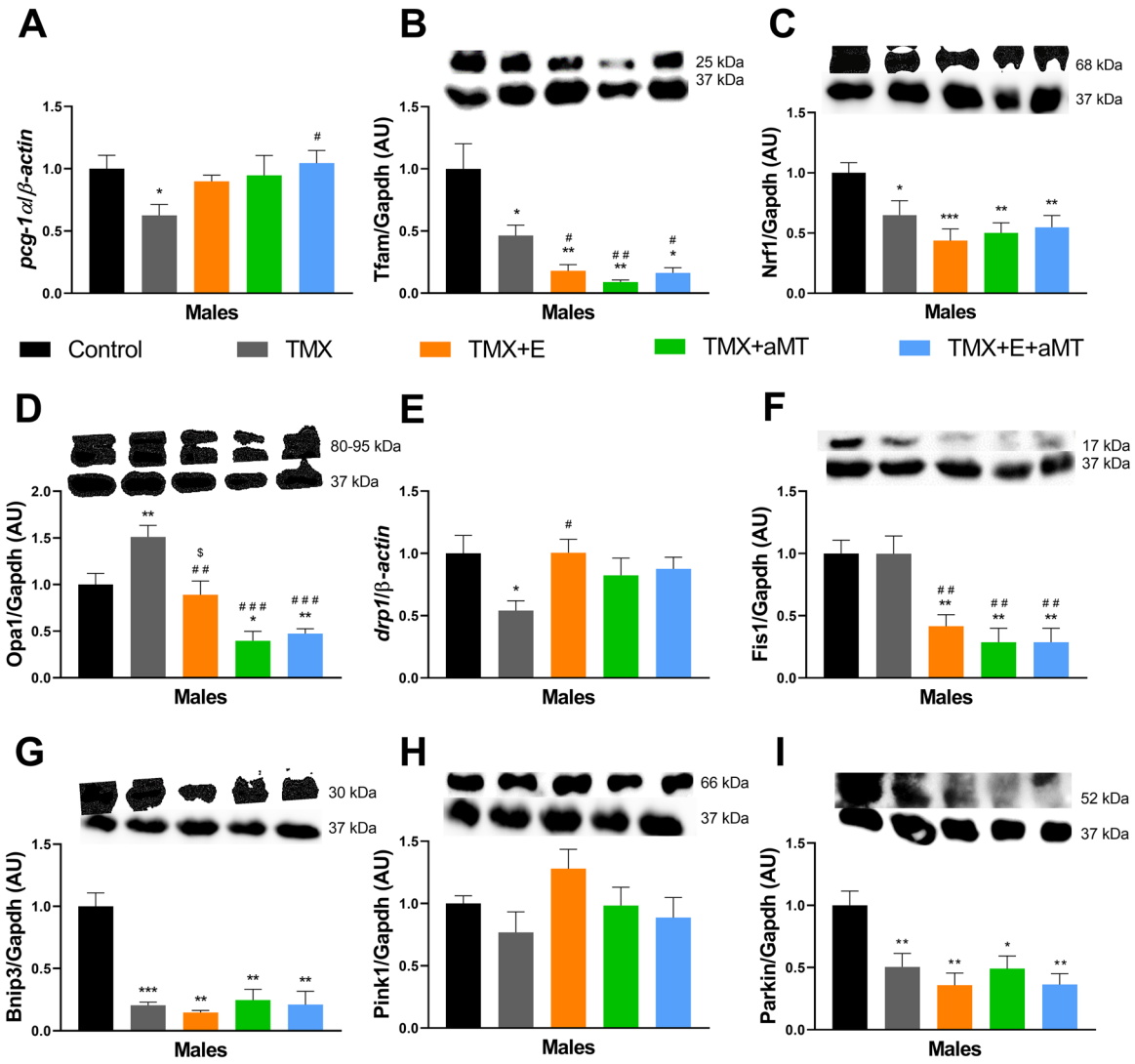

FIGURE 4 | Analysis of genes and proteins involved in mitochondrial dynamics in the iMS-*Bmal1*$^{-/-}$ mice and their controls. Regarding mitochondrial biogenesis, were analyzed: (A) Expression of *pgc-1α* mRNA; (B) levels of Tfam protein; and (C) levels of Nrf1 in control and experimental groups. Fusion and fission markers were, respectively: (D) Opa1 protein levels in the experimental groups; (E) *drp1* mRNA expression; and (F) Fis1 protein levels in the experimental groups. In mitophagy, the following were studied: (G) Bnip3 protein levels; (H) Pink1 protein levels; and (I) levels of Parkin in the same experimental groups. Data are expressed as means ± SEM ($n = 7$ animals/group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus TMX. $^{\$}p < 0.05$ versus TMX + E+aMT.

controlling the transition of mitochondria between low and high OXPHOS states [4, 13]. Here, we examined key components of this pathway. As expected, Bmal1 protein were practically absent in TMX animals and in those treated with exercise and/or melatonin (Figure 5A). Conversely, Clock protein levels significantly increased in the absence of *Bmal1*, but they were restored following treatments (Figure 5B). In addition, NAMPT protein levels decreased significantly not only in TMX but also in the treated groups, indicating the dependence of the former on *Bmal1* (Figure 5C). As expected, the NAD^{+}/NADH ratio decreased significantly in TMX animals, whereas treatments, mainly melatonin, recovered this ratio in muscle (Figure 5D).

The mRNA expression and protein levels of SIRT1 and SIRT3 were also measured in the experimental groups. Although *sirt1* expression remained unchanged in *Bmal1*-deficient GM, it increased in response to treatments, mainly with melatonin (Figure 5E). Despite this, Sirt1 protein levels were notably lower in TMX animals and in treated groups (Figure 5F). Conversely, *sirt3* expression increased in all experimental groups independent of *Bmal1* status (Figure 5G). Although Sirt3 protein levels were significantly higher in TMX animals, treatments reduced them below control (Figure 5H).

3.5 | iMS-*Bmal1*$^{-/-}$ Mice Exhibit Alterations in Antioxidant Defense, Inflammatory Response, and the FGF21-Klotho Pathway

Oxidative stress and inflammation, which increase with age, are key factors associated with sarcopenia. Accordingly, several proteins involved in these pathways, including FGF21-Klotho axis, were explored in our mouse model. In relation to antioxidant defense, the levels of Nrf2 protein were significantly reduced in the absence of *Bmal1* and recovered by melatonin, while exercise led to partial recovery (Figure 6A). TNf-α, a

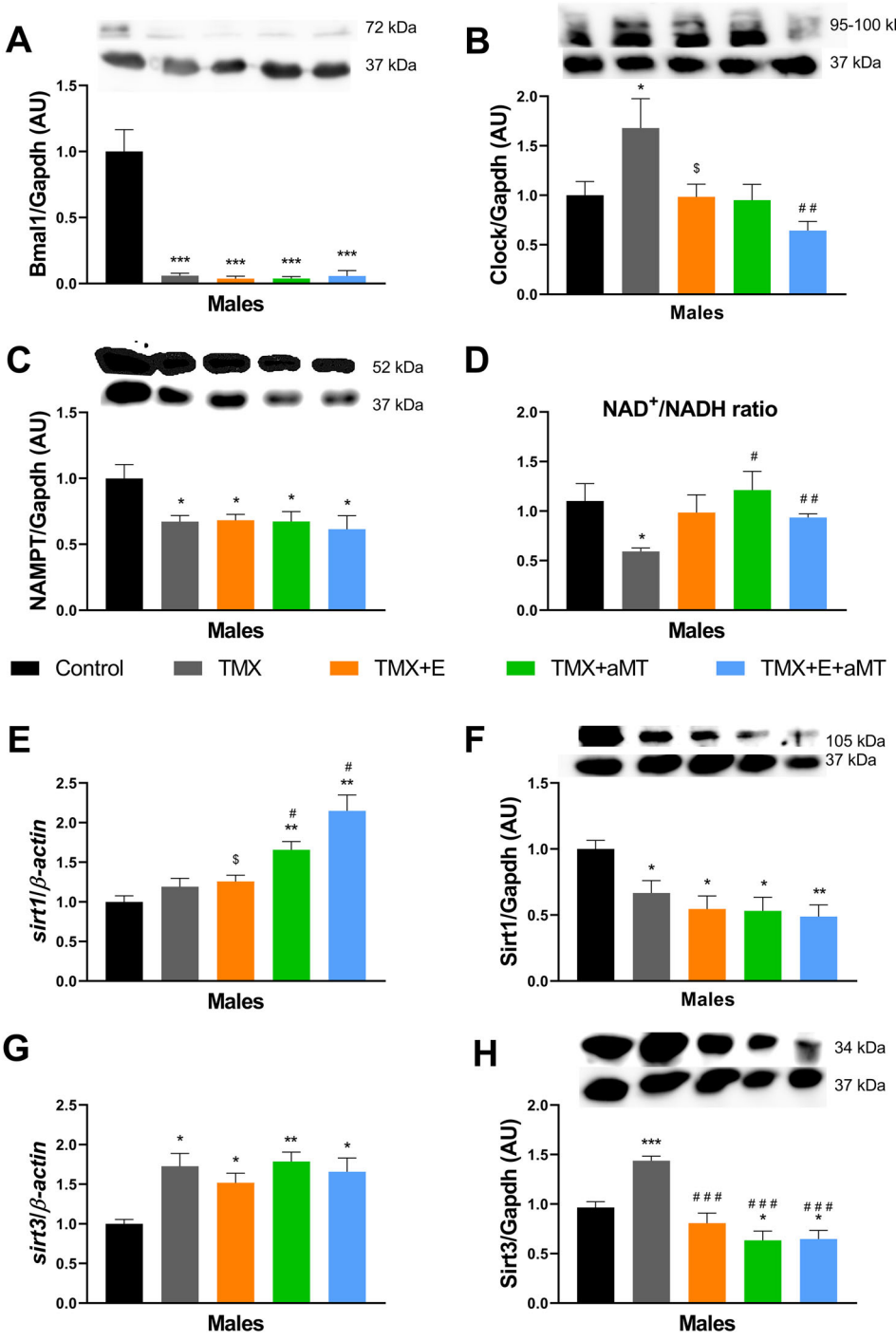

FIGURE 5 | Analysis of the genes and proteins involved in NAMPT-NAD$^+$-SIRTs axis. (A) Bmal1 protein levels; and (B) Clock protein in the control and experimental groups. (C) NAMPT protein levels; and (D) NAD$^+$/NADH ratio. (E) *sirt1* mRNA expression; and (F) Sirt1 protein levels in the experimental groups. (G) *sirt3* mRNA expression; and (H) Sirt3 protein levels in the same experimental groups. Data are expressed as means ± SEM ($n = 7$ animals/group). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus TMX. $^{\$}p < 0.05$ versus TMX + E+aMT.

marker of inflammation, decreased in mice with TMX and with the treatments, suggesting its dependency on *Bmal1* (Figure 6B). Given its association with longevity and its proposed role as a marker of sarcopenia [35, 36], the levels of klotho and fibroblast growth factor 21 (FGF21) were measured in iMS-*Bmal1*$^{-/-}$ mice. Klotho levels remained unchanged in TMX, but they increased in the TMX + E+aMT group compared to the control (Figure 6C). In contrast, FGF21 levels decreased with the loss of *Bmal1* in muscle, and the treatments were unable to restore its levels (Figure 6D).

4 | Discussion

Building on the understanding that the iMS-*Bmal1*$^{-/-}$ mouse model identified *Bmal1* deficiency as a critical factor driving

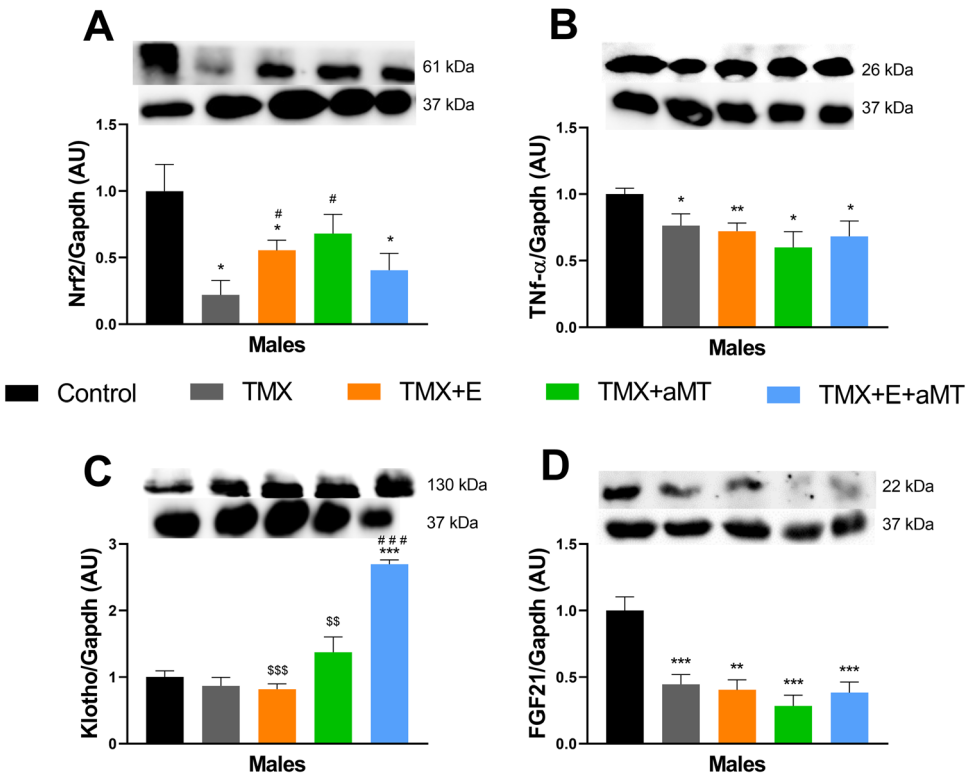

FIGURE 6 | Analysis of proteins involved in inflammatory pathways and FGF21-Klotho pathway in the iMS-*Bmal1*$^{-/-}$ mice and their controls. Inflammatory markers: (A) Nrf2; and (B) TNf-α protein levels. FGF21-Klotho pathway: (C) Klotho; and (D) FGF21 protein levels. Data are expressed as means ± SEM ($n = 7$ animals/group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus TMX. $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ versus TMX + E+aMT.

sarcopenia [21], the results presented here provide novel insights into the molecular mechanisms behind muscle deterioration in the absence of muscle *Bmal1*, thus identifying the role of intrinsic *Bmal1* in skeletal muscle homeostasis. An overall interaction between melatonin/exercise in the skeletal muscle pathways here analyzed is depicted in the Figure 7.

The circadian molecular clock plays a vital role in regulating the expression of key muscle genes involved in transcription, myogenesis, and metabolism [37]. Myogenesis declines with age, thereby contributing to muscle mass loss [1, 38]. In this study, we demonstrated that *Bmal1* deficiency in the GM results in reduced expression of MuSC markers and MRFs, indicating impaired muscle regeneration. Using a whole-body *Bmal1* knockout model, other authors demonstrated that its absence significantly impairs muscle regeneration, establishing this gene as a positive regulator of the myogenic response by promoting MuSC expansion during muscle repair. Additionally, its absence resulted in reduced muscle mass and a notable shift towards smaller muscle fibers [12, 39]. Interestingly, although these changes were expected to correlate with increased *mstn*, a factor associated with muscle mass loss [32], we observed lower expression of this myokine in the *Bmal1*-deficient model. These findings are consistent with those of Schroder et al., suggesting that *Bmal1* may regulate *mstn* expression, with alternative mechanisms contributing to the muscle damage observed in the absence of *Bmal1* [40]. Moreover, the alterations observed in iMS-*Bmal1*$^{-/-}$ mice revealed changes in the morphometric structure of the muscle, including reductions in both transverse length and CSA, which are linked to aged muscle [20, 41]. These findings strongly support that *Bmal1* deficiency in skeletal muscle primarily affects myogenesis and may explain the observed reduction in muscle fiber size and resulting atrophy in the GM, as previously documented in this animal model [21].

Mitochondria are essential organelles involved in cell structure and function, and their dysfunction, particularly reduced OXPHOS, is associated with skeletal muscle diseases like sarcopenia [2, 42]. Despite previous reports of structural damage and reduced mitochondrial number in iMS-*Bmal1*$^{-/-}$ mice [21], no significant differences in OCR were observed here in TMX-treated animals. However, our previous findings confirmed that *Bmal1* deficiency compromises CII functionality, as evidenced by decreased SDH activity, emphasizing the selective vulnerability of this complex [21]. Interestingly, CI inhibition significantly reduced OCR, suggesting that it may indirectly affects CII and compensates for this damage. These results underscore the essential role of *Bmal1* in maintaining mitochondrial integrity and respiration efficiency and may reflect compensatory metabolic adaptations in which increased CI activity helps sustain overall mitochondrial function despite the impairment of CII. Such adaptations are critical for maintaining cellular energy homeostasis under stress, as described in recent studies on mitochondrial energetic responses, where alterations in mitochondrial processes ensure cellular fitness [43].

Mitochondrial dynamics are also disrupted during aging, and *Bmal1* is a key gene responsible for maintaining mitochondrial integrity within the muscle's circadian clock [44, 45]. In this study, we show how the lack of *Bmal1* disrupts mitochondrial

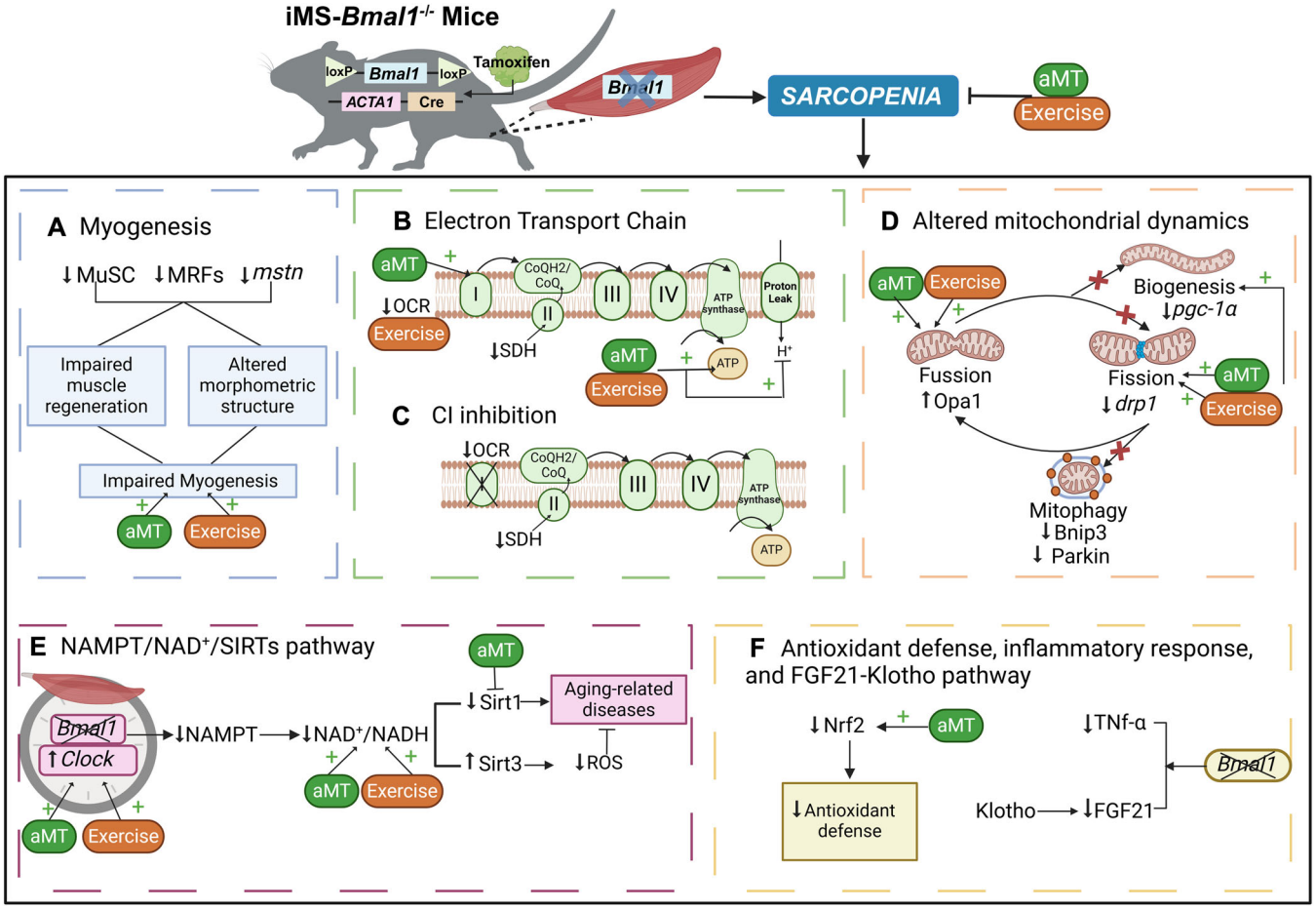

FIGURE 7 | Molecular mechanisms underlying *Bmal1* deficiency-induced muscle deterioration and sarcopenia: Protective effects of exercise and/or melatonin (aMT) treatments. (A) iMS-*Bmal1*$^{-/-}$ mice display reduced expression of satellite cell markers (MuSC), muscle regulatory factors (MRFs), and myostatin (*mstn*), resulting in impaired muscle regeneration and altered morphometric structure, which are restored by exercise and/or melatonin treatments; (B, C) iMS-*Bmal1*$^{-/-}$ mice exhibit reduced succinate dehydrogenase (SDH) activity in complex II (CII); however, melatonin enhances respiration through complex I (CI), increases ATP production, and decreases proton leak. Although exercise reduces OCR, it promotes ATP production and minimizes proton leak through alternative mechanisms; (D) iMS-*Bmal1*$^{-/-}$ mice show increased mitochondrial fusion along with reduced fission, biogenesis, and mitophagy. Exercise and/or melatonin treatments effectively restore the altered mitochondrial dynamics; (E) the absence of *Bmal1* in skeletal muscle leads to an increase in Clock, as well as decreased NAMPT levels and a reduced NAD$^+$/NADH ratio. The decline in Sirt1 is associated with the onset of aging-related diseases, such as sarcopenia, while the increase in *sirt3* serves as a compensatory mechanism to mitigate skeletal muscle damage. Exercise and/or melatonin restore Clock levels and the cellular NAD$^+$/NADH ratio; however, only melatonin is capable of upregulating *sirt1* expression; and (F) iMS-*Bmal1*$^{-/-}$ mice exhibit reduced antioxidant defenses, which are only counteracted by melatonin treatment. Both TNf-α and FGF21 levels are *Bmal1*-dependent.

dynamics with a reduction in mitochondrial biogenesis, fission, and mitophagy, along with enhanced mitochondrial fusion compared to controls. Our results are comparable with other studies reporting that *Bmal1*-deficiency affects mitochondrial dynamics in muscle [45] and other tissues [46, 47]. This *Bmal1*-mediated mitochondrial regulation clarifies changes, including altered morphology, increased mitochondrial size and damage, and reduced mitochondrial number, previously observed in GM and reinforces the notion that *Bmal1* is necessary for maintaining proper mitochondrial function in skeletal muscle [21].

Besides mitochondria, the circadian gene network, along with the NAMPT/NAD$^+$/SIRTs pathway, plays a key role in regulating energy metabolism [48]. Specifically, the Bmal1:Clock complex promotes NAMPT expression, which is critical for the synthesis of NAD$^+$, the substrate of sirtuins including SIRT1 and SIRT3 [13, 49]. As expected, we confirmed the compensatory increase in Clock protein levels in the GM of TMX-treated animals, confirming the data elsewhere published in this model [40, 50]. The loss of *Bmal1* in GM results in decreased NAMPT levels and subsequent drop in the NAD$^+$/NADH ratio, which has been linked to mitochondrial dysfunction and reduced muscle strength [51]. The reduction in Sirt1 protein has been associated with aging-related diseases [52], highlighting the role of *Bmal1* in maintaining Sirt1 stability and function during aging. On the other hand, it has been observed that *sirt3* expression increases in response to mitochondrial stress to help reduce excess ROS levels [53]. In contrast, elevated *sirt3* levels could potentially compensate for the absence of *Bmal1* in our model of acute skeletal muscle sarcopenia.

Age-dependent chronodisruption and inflammaging share common pathways, and the former may trigger inflammation because

Bmal1 plays a key role in regulating the immune system, antioxidant defense, and anti-inflammatory response [10, 54]. In this context, Nrf2, TNf-α, FGF21, and its coreceptor klotho are involved in these pathways and are associated with the frailty phenotype [35, 55, 56]. iMS-*Bmal1*$^{-/-}$ mice exhibited low levels of Nrf2, suggesting a diminished antioxidant response and increased susceptibility to inflammation and oxidative stress [57, 58]. While elevated levels of TNf-α and FGF21 are typically associated with inflammation, as well as reduced muscle mass and strength [59, 60], our model showed a decrease in both proteins, despite unchanged levels of klotho. This reduction could be attributed to their dependence on *Bmal1* for proper cellular function.

Although current treatments are insufficient to fully prevent or reverse sarcopenia, exercise and melatonin have demonstrated efficacy as therapeutic strategies independently of the presence of *Bmal1* [21]. Both exercise and melatonin regulate molecular rhythms in skeletal muscle, and their disruption may contribute to sarcopenia [61]. While exercise provides multiple benefits for overall homeostasis [62], excessive intensity can lead to increased oxidative stress. In this context, melatonin has been shown to exert a protective effect on muscle tissue by mitigating oxidative damage and reducing inflammation markers [63]. Emerging evidence suggests that exercise may influence melatonin secretion, possibly through the activation of the sympathetic nervous system and the subsequent increase in norepinephrine release, a key regulator of melatonin synthesis [64]. Therefore, the combined effects of melatonin and exercise may have a synergistic impact, optimizing physical performance and muscle homeostasis. However, further research is needed to fully elucidate the interaction between these factors and their effects on skeletal muscle physiology [63].

In this study, exercise and melatonin restored impaired myogenesis in iMS-*Bmal1*$^{-/-}$ mice, consistent with the effect of melatonin increasing the myogenic potential of MuSC and reducing fibrosis [65]. Additionally, exercise regulates the expression of core clock components and MyoD, both essential for maintaining skeletal muscle function [66]. These treatments also reversed morphometric alterations in the GM of *Bmal1*-deficient mice [21]. Interestingly, exercise reduced OCR in this model, reflecting potential mitochondrial stress or damage, as previously observed via transmission electron microscopy (TEM) [21], whereas melatonin improved OCR, which accounts for its ability to increase the activity of CI [67]. These findings suggest that CI is a key therapeutic target for reducing mitochondrial impairment, with melatonin offering a protective intervention. Surprisingly, both treatments reduced proton leak while increasing ATP production. This effect might be explained because although exercise generates ROS, it also induces physiological adaptations leading to improved mitochondrial efficiency and cellular health [68]. The benefits of melatonin and exercise extend to supporting healthy mitochondrial dynamics, as both are known to enhance mitochondrial efficiency [20, 69] and reverse mitochondrial alterations [21, 25]. Furthermore, melatonin and exercise treatments improved the metabolic state of iMS-*Bmal1*$^{-/-}$ mice by increasing the NAD^+/NADH ratio preserving mitochondrial NADH [48, 70]. It is also well known that melatonin induces *Sirt1* overexpression to protect against metabolic stress, supporting overall health and delaying the aging process [71]. Additionally, melatonin can prevent mitochondrial dysfunction by activating the SIRT1-PGC-1α-SIRT3 signalling pathways, promoting mitochondrial biogenesis in cardiac muscle [72], which may explain the changes observed in our mouse model. However, despite the increased NAD^+/NADH ratio and the metabolic improvements, the decrease in SIRT1 and SIRT3 levels suggests lower activity of these deacetylases. This could be due to other regulatory factors beyond NAD^+/NADH, such as posttranslational modifications and protein-protein interactions, which can modulate sirtuin activity [73]. Additionally, the metabolic state of *Bmal1*-deficient muscle could contribute to this phenomenon. Lower metabolic rates and mitochondrial dysfunction, commonly associated with *Bmal1* loss, may lead to impaired sirtuin stability or activity [52, 53], further contributing to the observed decrease in SIRT1 and SIRT3 levels.

Finally, although the treatments did not affect FGF21 levels, Klotho levels remained independent of *Bmal1* and increased in response to the combination of both treatments. This finding is consistent with previous studies indicating that both interventions independently enhance Klotho levels by upregulating its expression, thereby providing antiaging benefits [74, 75]. Interestingly, we observed an inverse relationship between Klotho and FGF21 during the treatments. While Klotho increased, FGF21 was not restored. This may be since Klotho is independent of *Bmal1* in our model, while FGF21 depends on *Bmal1* in muscle. This suggests that Klotho and FGF21 may have distinct or even opposing roles in regulating metabolic and aging processes, warranting further investigation to understand their interaction. Based on these findings, this study clarifies the mechanisms by which *Bmal1* deficiency triggers molecular changes leading to muscle deterioration and sarcopenia. Moreover, this study reaffirms that treatments with exercise and/or melatonin mitigate skeletal muscle damage associated with the loss of *Bmal1* (Figure 7). These results underscore the potential importance of conducting a clinical trial in humans to evaluate the effectiveness of these interventions in preventing and slowing the progression of sarcopenia.

Author Contributions

Darío Acuña-Castroviejo took responsibility for the study design; Yolanda Ramírez-Casas, José Fernández-Martínez and Alba López-Rodríguez performed the experiments; María Martín-Estebané Paula Aranda-Martínez, Sergio Esquivel-Ruiz, and Yang Yang analyzed the data; Germaine Escames, Yolanda Ramírez-Casas and José Fernández-Martínez prepared the manuscript, and Darío Acuña-Castroviejo critically revised the manuscript. All the authors participated in drafting the manuscript and approved the final version for publication.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.