

The Circadian Deficit in Sleep Deprivation: How Bmal1 Downregulation Fosters a Substrate for Atrial Fibrillation via Calcium and PI3K-AKT Pathways

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Abstract: Atrial fibrillation (AF) represents a major global health burden, and sleep disturbance is increasingly considered as a significant risk factor. However, the molecular mechanisms linking sleep disruption to AF remain incompletely understood. This study investigated the role of the core circadian clock gene Bmal1 in sleep deprivation-induced AF susceptibility. By conducting a differential gene analysis on the atrial fibrillation GSE79768 database and the sleep deprivation GSE98566 database, we found that Bmal1 was downregulated in the intersecting differential genes of these two databases, with PI3K-AKT pathway enriched among the common differential genes. Using mouse REM sleep deprivation models, we found that sleep deprivation significantly reduced Bmal1 expression in atria at both mRNA and protein levels. This reduction was associated with dysregulation of key calcium-handling proteins, characterized by a decrease in sodium +/ calcium+ exchanger 1 (NCX1) and Serca2a and an increase in the expression of Ryanodine receptor 2 (RyR2), which promotes a calcium overload arrhythmic substrate. Concurrently, sleep deprivation enhanced the phosphorylation of PI3K and Akt, suggesting the activation of this pathway. In vitro experiments on cardiomyocytes in neonatal mouse cardiomyocytes confirmed a causal relationship: Bmal1 knockdown replicated the calcium-handling protein dysregulation and increased PI3K/Akt phosphorylation observed in sleep-deprived mice, whereas Bmal1 overexpression exerted opposing effects. Histological analysis revealed an increase in atrial fibrosis in sleep-deprived mice. These findings delineate a novel pathway in which sleep deprivation downregulates atrial Bmal1, causing abnormal calcium homeostasis via altered NCX1 and RyR2/Serca2a expression and activation of the PI3K-Akt signaling pathway, thereby increasing AF susceptibility. The emergence of Bmal1 as a potentially key mediator in sleep deprivation-related AF provides insights for future therapeutic strategies.

Keywords: Atrial Fibrillation; Sleep Deprivation; Bmal1; Calcium Handling Proteins; PI3K/Akt Signaling pathway

1 Introduction

Atrial fibrillation (AF) is considered a mounting public health crisis world-

wide, with high morbidity and mortality. The severe detriment of this disease is starkly illustrated by its association with a significantly reduced survival rate, independent of other comorbid conditions, underscoring the urgent need for effective interventions. The adverse impact of AF manifests through several interconnected pathways. First of all, its etiology is remarkably complex and multifactorial, involving genetic predisposition, structural remodeling, electrical triggers, and so on. This intricate pathogenesis makes early diagnosis exceptionally challenging. Secondly, the chaotic quivering of the atria leads to hemodynamic stasis, which dramatically increases the risk of thrombosis. The thrombus can cause devastating systemic events, with ischemic stroke being the most life-threatening consequence. Furthermore, the development and progression of AF are driven by a complex network of molecular signaling pathways and regulatory circuits, including calcium-handling abnormalities, inflammatory cascades, and fibrotic remodeling, which have not been fully understood. Even after decades of intensive research, the exact mechanisms of the onset and development of AF remain incompletely elucidated, providing fertile ground for further exploration. Intriguingly, new clinical findings suggest a distinct circadian pattern in the emergence of AF, with a higher frequency of episodes occurring during the morning and evening hours, hinting at a possible involvement of the autonomic nervous system and circadian biology in its pathophysiology, which is an intriguing field ripe for further investigation.

The process of sleep is intricate. The heart rhythm during sleep is influenced by a wide range of pathological conditions, including the imbalance in the autonomic nervous system. The fast-paced life in modern society and increasing daily stress have led to people's neglecting of sufficient sleep. Globally, sleep problems, including obstructive sleep apnea (OSA), sleep deprivation or restriction, and insomnia, are becoming an increasingly recognized issue in the general population. The growing disconnect between sleep needs and actual sleep attainment has exerted an adverse influence on mental health, body metabolism, the immune system, and cardiovascular health.

Epidemiological researches have suggested a potential rise in AF cases due to sleep disruptions. A 2018 American meta-analysis showed a combined odds ratio of 1.70 (95% CI, 1.40–2.06, $I^2 = 0$) for recurring AF in OSA patients compared with those without OSA. In 2022, a study led by Zhenni Chen in China reaffirmed the significant association between sleep-related breathing issues and the rising probability of postoperative AF in heart surgery patients (OR 1.49; 95% CI, 1.30–1.70; $p < 0.001$). Negar Morovatdar also discovered a link between shorter sleep durations or insomnia and a heightened risk of AF (HR = 1.58 and HR = 1.33, respectively).

However, the process linking sleep disturbances to atrial fibrillation remains somewhat unclear. Current research indicates potential impacts on heart structure and electrical remodeling due to oxidative stress, inflammation, and alterations in autonomic nervous system functions. However, the fundamental molecular pro-

cesses still require further investigation.

Regarding the mechanism of atrial fibrillation, scholars including Dobromir Dobrev pointed out that Ca^{2+} overload and the decrease in $\text{I}_{\text{Ca,L}}$ can trigger atrial fibrillation. Calcium ion (Ca^{2+}) influx is the most important ionic current during cardiac contraction. In cardiac muscle cells, calcium ions mainly enter the cells through voltage-gated L-type calcium ion channels (LTCCs; Cav 1.2 subtype), which are located in T-type tubules, forming an inward calcium ion current ($\text{I}_{\text{Ca,L}}$), and subsequently promoting the release of a large amount of calcium ions from the sarcoplasmic reticulum (SR) through RyR2 (ryanodine receptor 2). During diastole, intracellular calcium ions mainly return to baseline levels through the reuptake of calcium ions by the SR calcium ion ATPase and through efflux via $\text{Na}^{+}/\text{Ca}^{2+}$ exchangers. The dysregulation of these calcium ion flows is the core cause of various cardiac and vascular diseases. Improper calcium ion handling in cardiac muscle cells is also one of the main causes of atrial fibrillation. Increasing evidence indicates that the PI3K/Akt signaling pathway plays a non-redundant role in the regulation of calcium ions. The downstream effectors of PKC (protein kinase C) and PI3K, PKB/Akt (protein kinase B), have regulatory effects on calcium ions. PKC is activated by calcium ions and diacylglycerol in the cytoplasm, and then phosphorylates a series of substrates related to the regulation of calcium ion flow, such as $\text{Na}^{+}/\text{Ca}^{2+}$ exchangers, TRPs (transient receptor potential channels), and PLC, etc.

Bmal1, a core component of the circadian clock system, belongs to the basic helix-loop-helix PER-ARNT-SIM (bHLH-PAS) family of transcription factors. It forms a heterodimer with CLOCK, serving as the primary positive regulator in the transcription-translation feedback loops (TTFLs) that drive circadian rhythmicity at the cellular level. This Bmal1/CLOCK complex activates the transcription of key circadian repressors, Period (Per) and Cryptochrome (Cry), which in turn feedback to inhibit its own activity, thereby generating 24-hour oscillations in gene expression. Bmal1 is indispensable for this autoregulatory loop and the rhythmic output of numerous genes governing metabolism and cellular homeostasis. Disruption of Bmal1 is closely linked to the pathogenesis of metabolic diseases, and its expression is notably reduced in the skeletal muscle of type 2 diabetic patients. Furthermore, Bmal1 plays a critical role in the heart. Cardiomyocyte-specific knockout of Bmal1 alters the expression of insulin signaling and metabolic factors. Notably, Bmal1 deficiency has been shown to activate the PI3K-Akt signaling pathway, thereby promoting cardiac remodeling, including cardiomyocyte hypertrophy and fibrosis. PI3K and Calcium Signaling in Cardiovascular Disease. This effect can be reversed by Akt inhibition. The PI3K-Akt pathway, particularly the $\text{PI3K}\alpha$ isoform, is a well-established regulator of cardiac L-type calcium channels (LTCCs)¹, influencing their membrane localization, stability, and calcium current ($\text{I}_{\text{Ca,L}}$), which is crucial for cardiac contraction and electrical stability¹⁰. While circadian rhythms in LTCC function have been observed, the direct regulatory relationship between the CLOCK-

BMAL1 heterodimer and cardiac LTCCs remains undefined. To date, no study has comprehensively investigated the changes in atrial Bmal1, calcium-handling proteins, and the PI3K-Akt pathway following sleep deprivation, nor their potential collective role in atrial fibrillation. Therefore, elucidating this mechanism is a significant and unresolved question in the field.

Given the above findings, we hypothesized that the circadian gene Bmal1 participates in the pathogenesis of AF by regulating the expression of calcium channels and intracellular calcium concentration in atrial myocytes through the PI3K-Akt signaling pathway. In the present study, by generating a mouse sleep deprivation model with MPPM method, and interfere the level of Bmal1 protein in cardiomyocytes, we found that the decrease of circadian gene protein Bmal1 in mouse atria and cardiomyocytes may promote atrial fibrosis, and influence Ca²⁺ handling proteins. Mechanically, we proved that sleep deprivation may affect Ca²⁺ handling proteins and PI3K-AKT pathway in atria, which are closely related to the occurrence of atrial fibrillation, by decreasing the level of circadian gene protein Bmal1. This may partly explain the mechanism of sleep disorders related AF, and provide new therapeutic methods for sleep disorders patients to prevent or alleviate AF.

2. Results

2.1. Identification of DEGs and Functional enrichment analysis of the co-DEGs of AF and SD

Based on GEO2R, we identified 5,344 upregulated and 4,545 down-regulated DEGs in the GSE79768 dataset related to AF, and a total of 2717 (935 up, 1782 down) sleep deprivation-related DEGs were obtained from GSE98566. The volcano plots of GSE79768 and GSE98566 were manifested in Fig. 1A and **Fig. 1B**, respectively. The 1473 overlapping genes between AF-related DEGs and sleep deprivation-related DEGs were shown in the Venn diagram (**Fig. 1C**). The list of the co-DEGs in “AF-GSE79768|SD-GSE98566” were listed in supplementary material (jVenn-AF79768&SD98566.csv), and ARNTL, another scientific name for BMAL1, were among the overlapping genes. The P value and logFC value of ARNTL in datasets GSE79768 and GSE98566 were shown in Table 1, and the original results of top differentially expressed genes in these two datasets were listed in supplementary material (GSE79768.top.table.tsv and GSE98566.top.table.tsv).

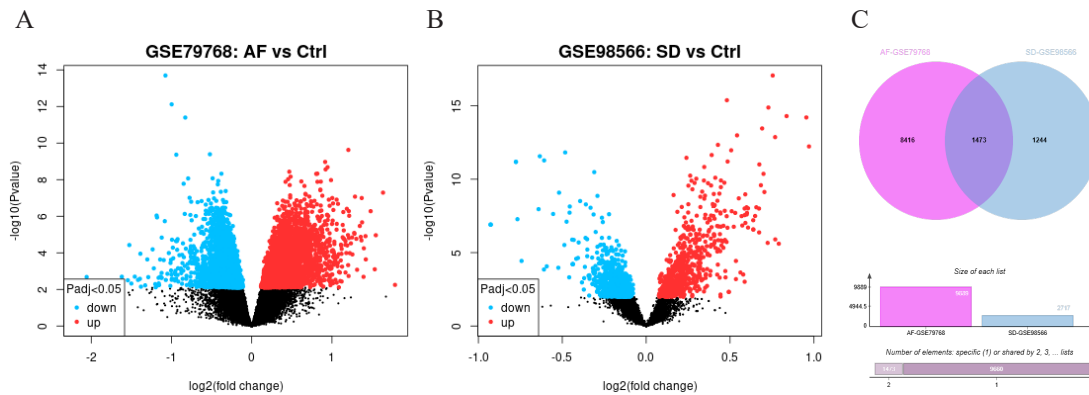
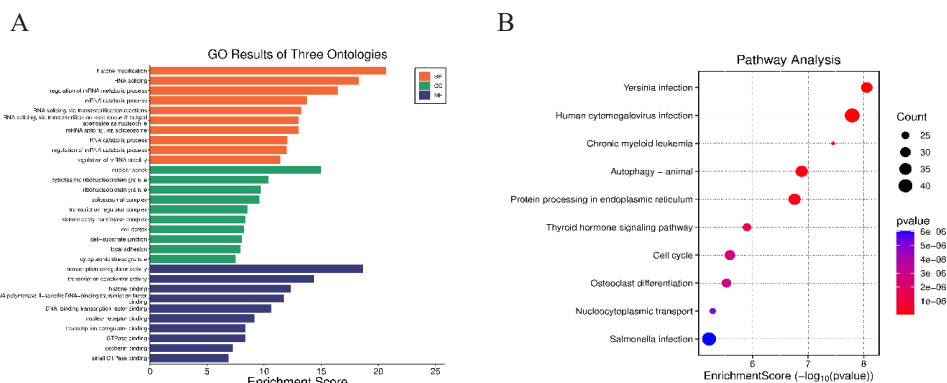


Figure 1. DEGs representation by volcano plot diagrams and Venn diagram. (A) The volcano plot of DEGs in GSE79768. (B) The volcano plot of DEGs in GSE98566. (C) Venn diagram of DEGs from GSE79768 and GSE98566. AF, atrial fibrillation; SR, sinus rhythm; SD, sleep deprivation; DEGs, differentially expressed genes.

Table 1. The P value and logFC value of ARNTL in datasets GSE79768 and GSE98566.

Dataset	adj.P.Val	P.Value	t	B	logFC	Gene.symbol
GSE79768	0.132	0.0362	-2.20	-4.11	-0.26678	ARNTL
GSE98566	0.0461	0.0089	-2.65	-3.10	-0.16332	ARNTL

GO and KEGG pathway analysis were performed to identify the potential biological functions of the common genes in SD and AF samples. In the GO analysis results, 558 biological process (BP) terms, 111 cellular component (CC) terms, and 86 molecular function (MF) terms were identified. P value < 0.05 signified threshold significance. Overall, 21 genes were primarily abundant in BP term “histone modification”, 15 genes were located in the “nuclear speck” of CC term, and 19 genes were abundant in the MF term “transcription coregulator activity” as indicated in **Fig. 2A**. Furthermore, the results of KEGG enrichment analysis indicated that the co-DEGs of AF and SD were implicated in several critical pathways, namely, autophagy, protein processing in endoplasmic reticulum, and thyroid hormone signaling pathway (**Figure 2B**). Notably, pathways including the main components PIK3 and AKT were also significantly enriched (**Table 2**). The pathwayview maps of representative KEGG pathways that including PI3K or AKT were showed in **Fig 2C-E**.



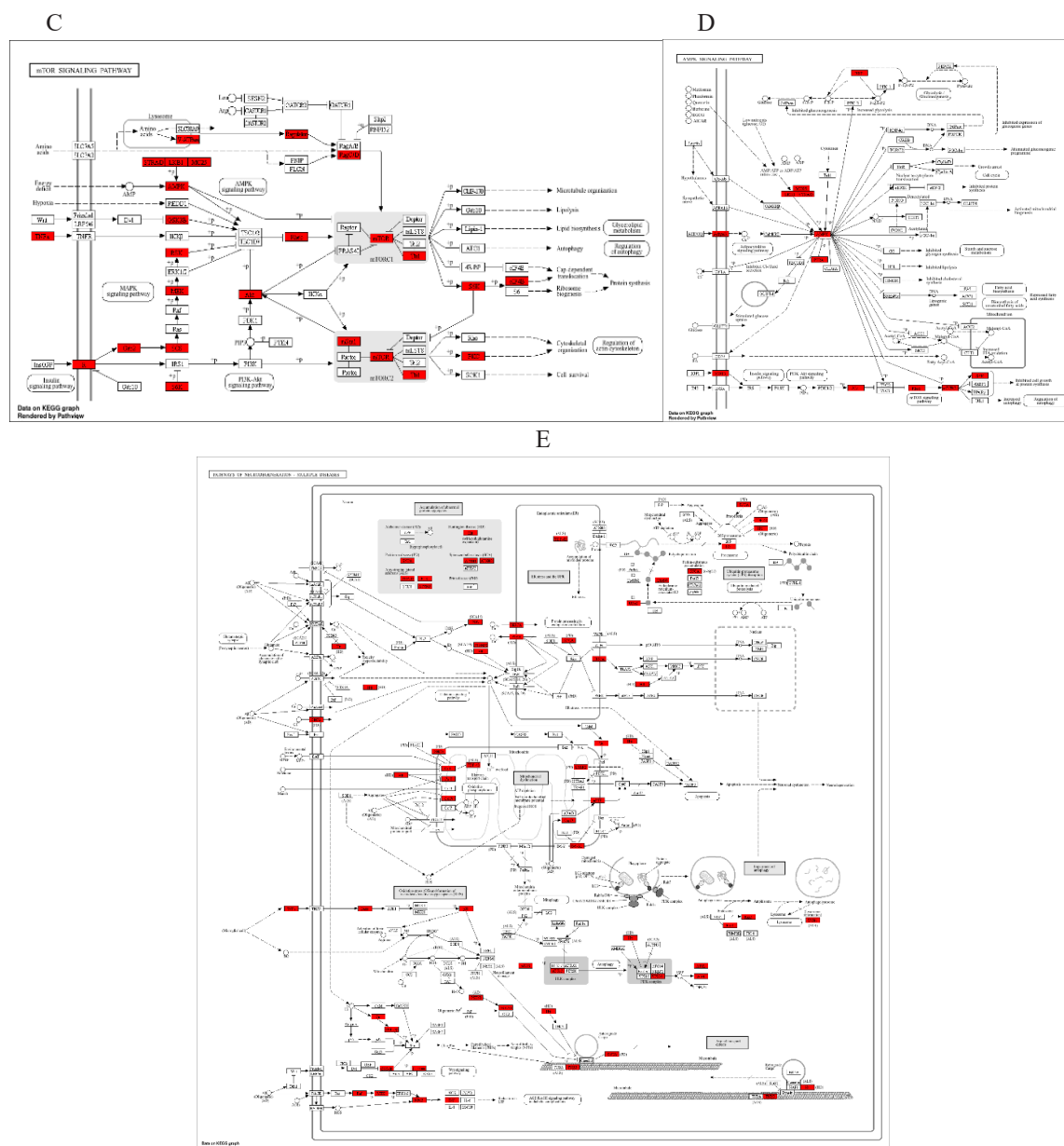


Figure 2. GO term and KEGG pathway analysis for DEGs significantly associated with AF and SD. (A) GO enrichment analysis of the 1473 key common genes of AF and SD. (B) The bubble diagram illustrates the top 10 entries for KEGG enrichment analysis of the 1473 common genes. (C) The pathview map of representative KEGG pathway hsa04150 mTOR signaling pathway. (D) The pathview map of representative KEGG pathway hsa04152 AMPK signaling pathway. (E) The pathview map of representative KEGG pathway hsa05022 pathways of neurodegeneration-multi-ple diseases.

Table 2. The basic information and enrichment scores of representative KEGG pathways that including PI3K or AKT.

ID	Description	GeneRatio	BgRatio	pvalue	p.adjust	geneID	Count	En-richme-nt. Score	Fold.En-ri-chment	
hsa04136		Autophagy - other	8/718	32/9396	0.0023	0.0124	PIK3C3/MTOR/etc	8	2.64598	3.271588
hsa04145	Phagosome	21/718	159/9396	0.0095	0.0365	PIK3C3/PIK-FYVE/etc	21	2.024227	1.728386	
hsa04150	mTOR signaling pathway	24/718	158/9396	0.0009	0.0065	MAPKAP1/MTOR/etc	24	3.05673	1.9878	
hsa04151	PI3K-Akt signaling pathway	35/718	362/9396	0.0870	0.2120	IL6R/MTOR/AKT2/etc	35	1.060423	1.265255	
hsa04152	AMPK signaling pathway	15/718	122/9396	0.0448	0.1251	MTOR/AKT2/etc	15	1.348845	1.608977	
hsa05022	Pathways of neurodegeneration - multiple diseases	47/718	483/9396	0.0494	0.1356	PIK3C3/SNCA/etc	47	1.30633	1.273413	

2.2. Sleep deprivation decreases the level of the circadian gene Bmal1 in mouse atrium

Brain and muscle arnt-like protein 1 (Bmal1), a crucial transcription factor, is in charge of regulating circadian rhythm and is involved in the occurrence of multiple heart diseases. Hence, to understand how the clock gene Bmal1 responds to sleep deprivation, we measured both the mRNA and protein expression levels of Bmal1 in the atrium using RT-qPCR and Western blotting. We found that, compared with the control group, both the mRNA and protein levels of Bmal1 decreased significantly in the sleep deprivation group. Atrial Bmal1 mRNA levels in sleep-deprived mice were reduced to 3.31% of the control values (Fig. 2G), while levels of Bmal1 protein in the atrium of mice exposed to sleep deprivation were reduced to 23.5% of the values from mice in the control group (Fig. 2A-B). The flowchart illustrates the design and workflow of the entire in vivo study (Fig. 1).

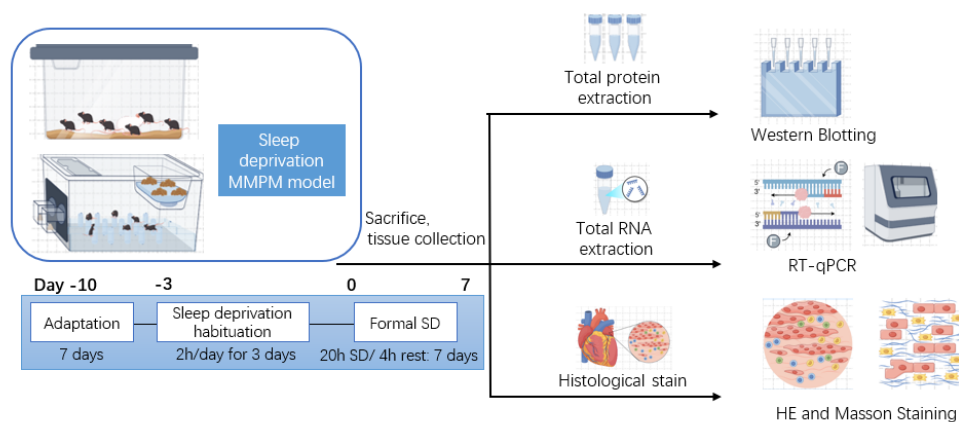


Figure 3. Workflow of the in vivo study. Mice were randomly divided into two groups after acclimatization for 7 days. Then, after 3 days of SD habituation (3 hours per day), the mice in SD group were subjected to SD for 7 consecutive

days (from 8 a.m. on day 0 to 8 a.m. on day 7, rest from 5 p.m. to 9 p.m. everyday). Tissue collection was carried out on day 7.

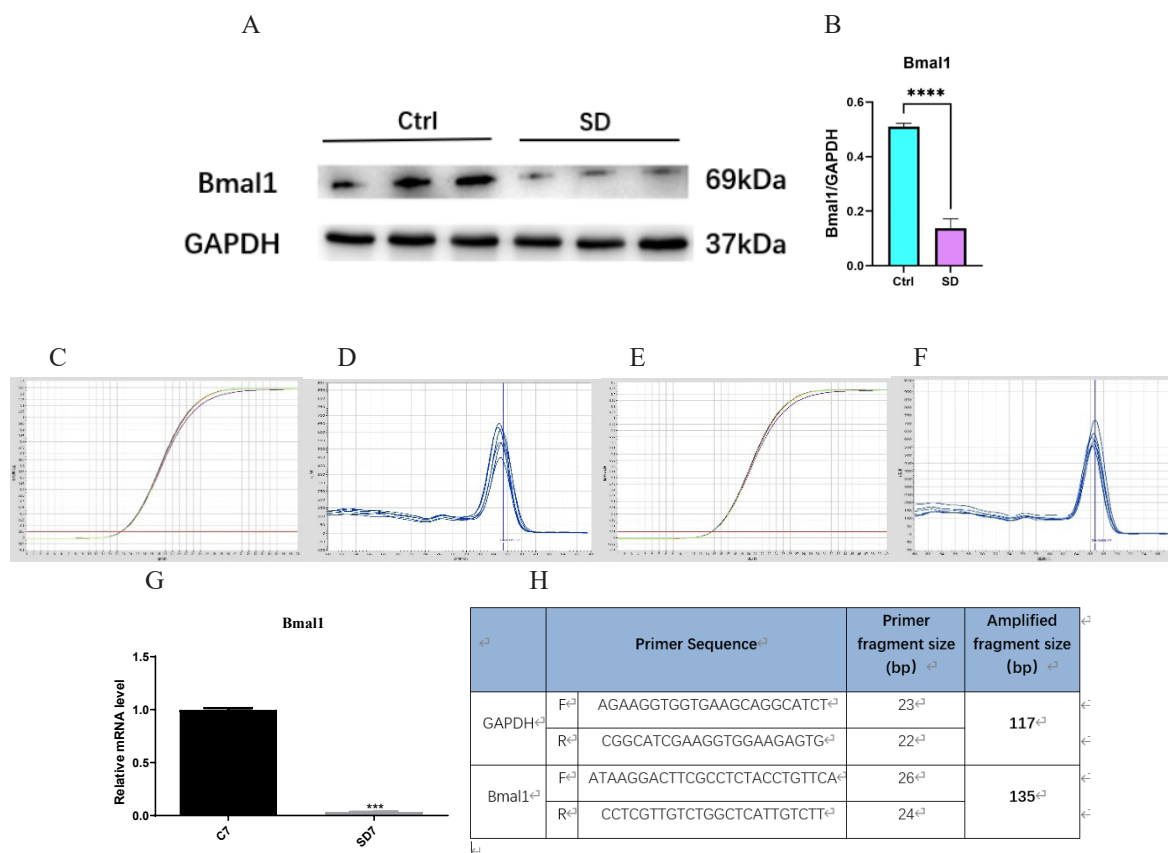


Figure 4. Acute sleep deprivation reduced the levels of Bmal1 protein and mRNA. (A) Original western blot bands of Bmal1 with GAPDH as loading control. (B) Analysis of Bmal1 protein levels in the two groups. (C) Original GAPDH amplified curve in RT-qPCR. (D) Original GAPDH melting curve in RT-qPCR. (E) Original BMAL1 amplified curve in RT-qPCR. (F) Original BMAL1 melting curve in RT-qPCR. (G) RT-qPCR showing the levels of Bmal1 mRNA in each group. (H) Primers used were listed.

2.3 Sleep deprivation alters Ca^{2+} handling protein levels in the mouse atrium

Atrial myocyte intracytoplasmic Ca^{2+} overload plays an essential role in the development of atrial fibrillation, and Ca^{2+} handling channels, including Cav1.2, NCX1, RyR2, and Serca2A, are closely associated with the formulation of calcium overload. We explored the relationship between calcium-handling channel proteins and acute sleep deprivation in 6 to 8-week-old male mice.

To evaluate the changes in the levels of calcium pumps in the sarcoplasmic reticulum after sleep deprivation, we measured the expression levels of RYR2, which is responsible for releasing Ca^{2+} into the cytoplasm, and Serca2A, which is involved in recapturing Ca^{2+} back into the sarcoplasmic reticulum. The results revealed that acute sleep deprivation significantly upregulated RyR2 expression and downregulated Serca2A (**Fig. 3C-D**), indirectly confirming its effective in-

duction of intracellular Ca^{2+} overloading.

To determine whether sleep deprivation affects calcium pumps on cytomembrane, we measured the level changes of NCX1 and Cav1.2. The level of the sodium-calcium exchanger responsible for pumping Ca^{2+} out of the cell was diminished significantly after acute sleep deprivation (**Fig. 3E-F**), also elevating the intracellular calcium level just like the alterations in RyR2 and Serca2A, whereas the level of Cav1.2 has no significant change (**Fig. 3A-B**). These results suggest that acute sleep deprivation significantly alter the levels of some Ca^{2+} handling proteins that could increase intracellular calcium levels in mouse atrium.

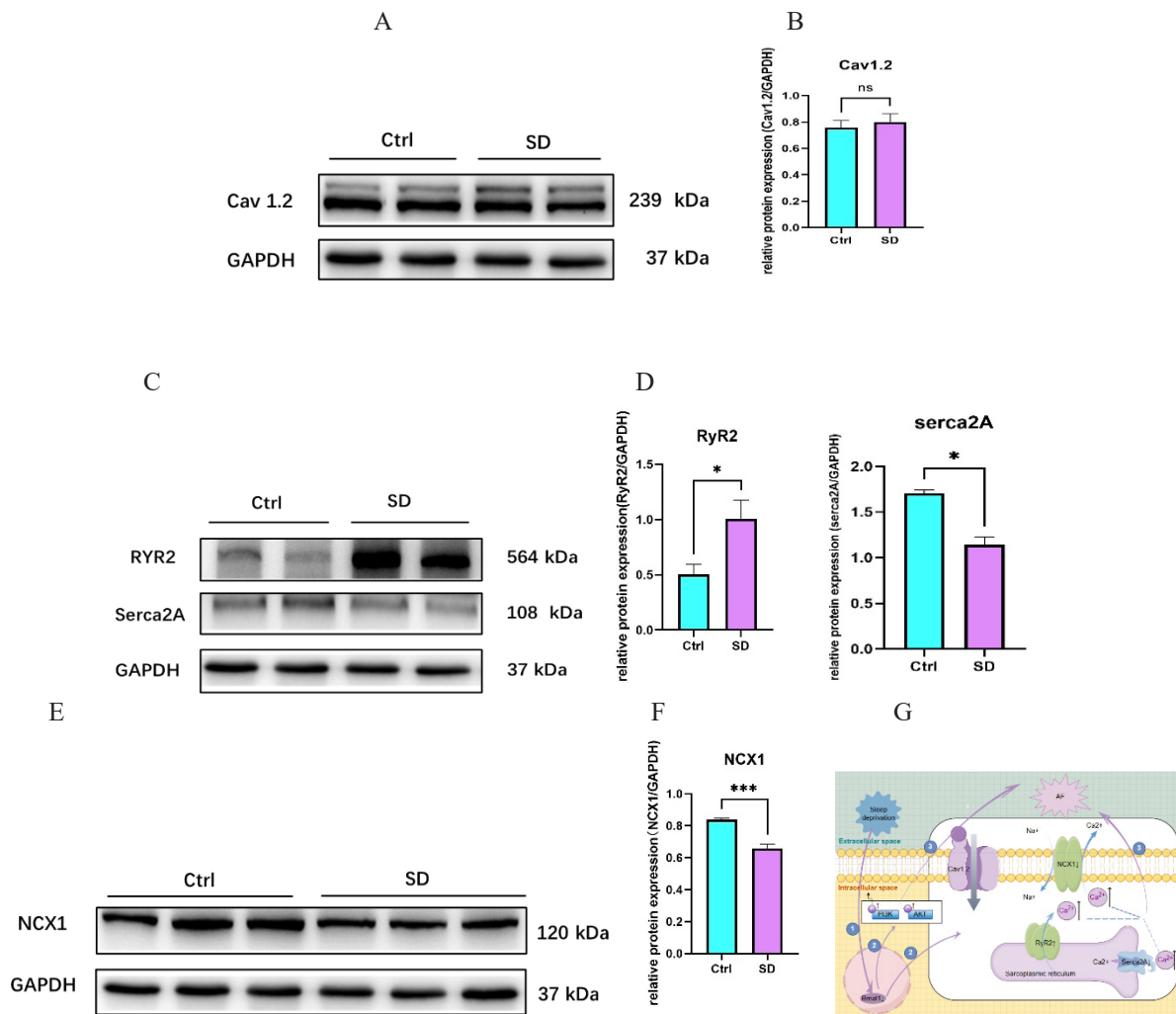


Figure 5. Changes in Ca^{2+} handling protein levels induced by sleep deprivation in mouse atria. (A, C, E) Original Western blot bands showing the effects of SD on the levels of Cav1.2, RyR2, SERCA2a and NCX1. (B, D, F) Relative protein expression of Cav1.2, RyR2, SERCA2a and NCX1 in the two groups. * $P < 0.05$, *** $P < 0.001$ ($n = 3$ Control, $n = 3$ Sleep Deprivation). (G) Diagram of the mechanism by which sleep deprivation regulates Ca^{2+} handling proteins by regulating circadian gene protein Bmal1.

2.4 Sleep deprivation aggravates the fibrosis of atrium in mice and interstitial edema

The histological examination of the atrial tissue showed unique structural changes following sleep deprivation. In the control group, hematoxylin and eosin (HE) staining revealed a well-organized myocardial structure, distinct cardiomyocyte layout, and intact interstitial spaces at both low (1x) and high (40x) magnification levels (**Fig. 5C**). Conversely, sleep-deprived mice exhibited signs of structural disorganization, including interstitial edema, irregular cardiomyocyte alignment, and occasional inflammatory infiltrates (**Fig. 5C**). Masson's trichrome staining further supported these findings, showing that there was a significant difference in the proportion of collagen between the two groups (**Fig. 5E**). There was only a slight collagen buildup in tissues in the control group, while the sleep-deprived group displayed noticeable increases in collagen fiber accumulation (**Fig. 5D**). Such alterations were consistently observed across multiple fields of view (**Fig. 5A-B**), suggesting that sleep deprivation may lead to significant atrial remodeling characterized by inflammatory changes and fibrotic progression.

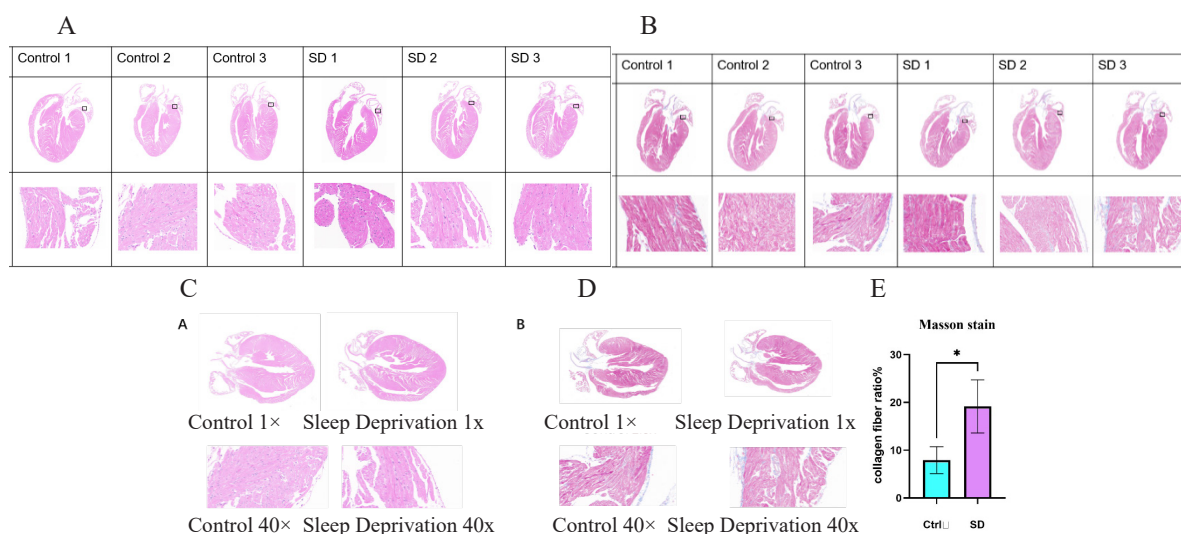


Figure 6. Sleep deprivation disorganized myocardial structure and increased atrial fibrosis. (A) The microstructure changes in hearts stained with H & E staining in control group and sleep deprivation group. (B) The fibrosis condition changes in hearts stained with Masson's trichrome staining obtained from the two groups of mice. (C, D) Representative section images of H & E and Masson's trichrome staining. Collagen and muscle fibers are stained in blue with Masson's trichrome staining(D). (E) Summarized data of collagen volume fraction in Masson's trichrome staining. * $P < 0.05$ ($n = 3$ Control, $n = 3$ Sleep Deprivation) .

2.5 Sleep deprivation increases the phosphorylation of the PI3K/Akt pathway

The PI3K/Akt pathway is important in the occurrence and development of atrial fibrillation. Studies have shown that the enhanced phosphorylation of this pathway is associated with an increase in atrial fibrosis, which is an important

substrate for atrial fibrillation. To explore whether sleep deprivation could induce AF through the PI3K/Akt pathway, we measured the total and phosphorylated protein expression levels of PI3K and Akt in mouse atrial tissues in the control group and the sleep deprivation group (Fig. 4) The results demonstrated that the phosphorylation ratios of both PI3K and Akt were increased after acute sleep deprivation (Fig. 4D, G), accompanied by the increase of pAKT and pPI3K (Fig. 4B, E) and the decrease of total AKT and total PI3K (Fig. 4C, F). Based on previous evidence from other studies and the higher activation rates of the PI3K/AKT signaling pathway in the SD mouse atria in our experiment, we speculate that the PI3K/AKT pathway plays a role in the impact of sleep deprivation on the increased fibrosis rate in the atria.

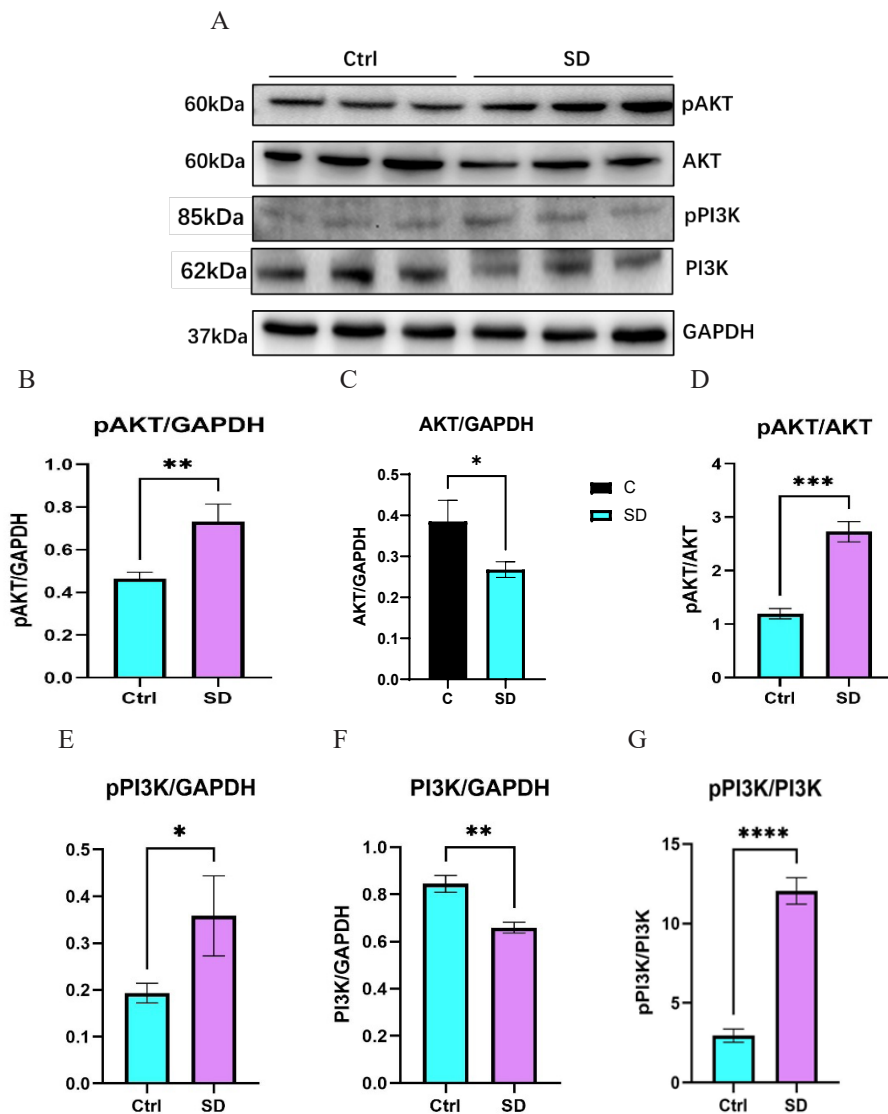


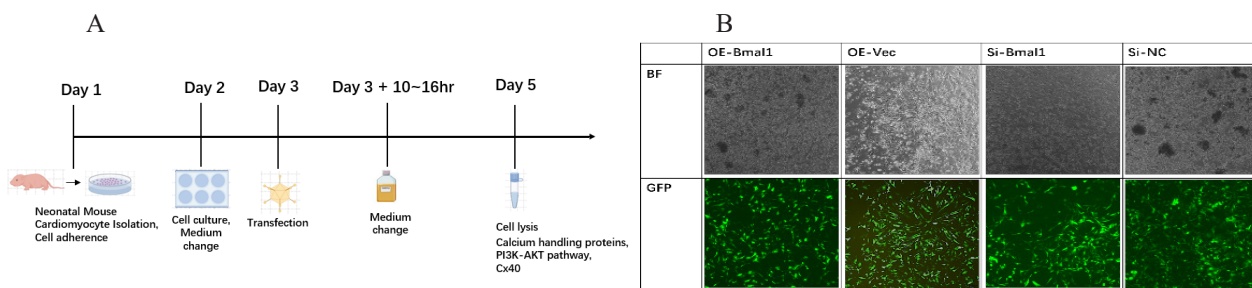
Figure 7. Sleep deprivation induced the activation (phosphorylation) of PI3K/Akt signaling pathway in mouse atria. (A) Original expression levels of total Akt, p-AktSer473, total PI3K, and p-PI3K in mouse atria after 7 days of consecutive sleep deprivation. (B) pAKT proteins in sleep deprivation group were significantly higher than those in control group. (C)

Relative protein expression of total AKT in the two groups with GAPDH. (E) pPI3K proteins in sleep deprivation group were significantly higher than those in control group. (F) Relative protein expression of total PI3K in the two groups with GAPDH. (D, G) Ratios of pAKT/AKT and pPI3K/PI3K in each group.

2.6 Knockdown of Bmal1 affects Ca^{2+} handling proteins and increases the phosphorylation of the PI3K/Akt pathway in cardiomyocytes in vitro

To examine whether Bmal1 plays a role in regulating Ca^{2+} handling proteins and PI3K/Akt pathway in atria, we generated in vitro Bmal1 knockdown model in neonatal mouse cardiomyocytes via adenovirus transfection. Protein levels were tested 48 hours later after the virus transfection (Fig. 6A). Representative fluorescence assay (Fig. 6B) and western blotting bands of Bmal1 levels in different groups (Fig. 6C-D) exhibit a high transfection efficiency of adenovirus in cardiomyocytes. The results revealed that downregulation of Bmal1 expression significantly increased RyR2 and diminished Serca2A in cardiomyocytes (Fig. 7B), which was in consistent with the trend of these two proteins after sleep deprivation in mouse atrium. Similarly, the level of NCX1 also decreased significantly in the Bmal1 knockdown group (Fig. 7C), revealing the same expression pattern as that in the sleep deprivation model. The alterations of Ca^{2+} -regulating proteins in cardiomyocytes following Bmal1 knockdown mirror the effects observed after sleep deprivation, further supporting the hypothesis that Bmal1 may be involved in the SD-induced intracellular Ca^{2+} overload. Intriguingly, Cav1.2 descended after Bmal1 knockdown, which could cause a decrease in L-Type calcium current and a shortening of APD, thereby triggering atrial fibrillation.

Then, we assessed the expression of PI3K/AKT signaling pathway in Bmal1 downregulation group and control group. It turned out that the level of total PI3K decreased significantly, while p-AKT increased after Bmal1 was knocked down (Fig. 8C). Analysis of phosphorylation rates in the two groups further demonstrated that both the ratio of pAKT/AKT and pPI3K/PI3K were elevated after Bmal1 was knocked down (Fig. 8D). In conclusion, Bmal1 knocking down not only regulates Ca^{2+} handling proteins but may also play a role in promoting the phosphorylation of PI3K/AKT pathway.



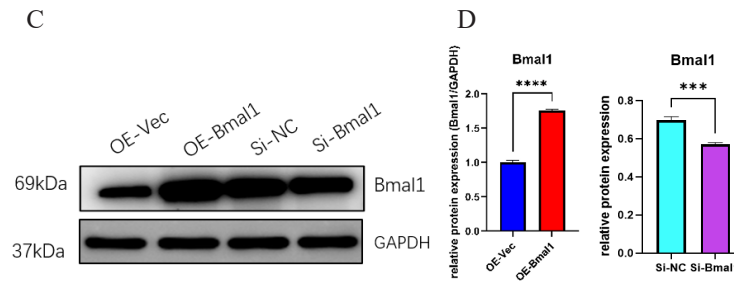


Figure 8. Bmal1 interference models were constructed using adenovirus. (A) Experimental design of the in vitro study. (B) Representative fluorescence assay exhibiting a high transfection efficiency of adenovirus in neonatal mouse cardiomyocytes. (C) Expression of Bmal1 proteins in Bmal1 overexpression empty vector control group, Bmal1 overexpression group, Bmal1 knockdown normal control group, and Bmal1 knockdown group in neonatal mouse cardiomyocytes. (D) Relative protein expression of Bmal1 in the four groups.

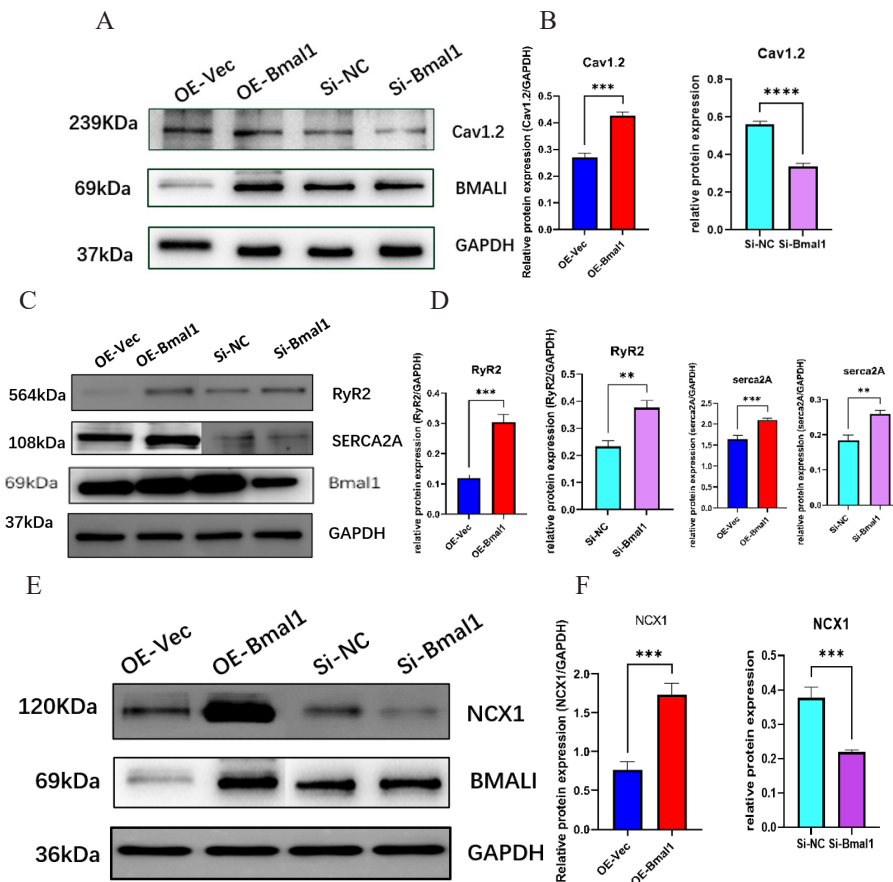


Figure 9. Bmal1 knockdown revealed the same expression pattern of Ca²⁺ handling proteins as that in the sleep deprivation model, while Bmal1 overexpression reversed them. (A, C, E) Original Western blot bands and showing the effects of Bmal1 overexpression and Bmal1 knockdown on the levels of Cav1.2, RyR2, SERCA2a and NCX1. (B, D, F) Relative protein expression of Cav1.2, RyR2, SERCA2a and NCX1 in the four groups.

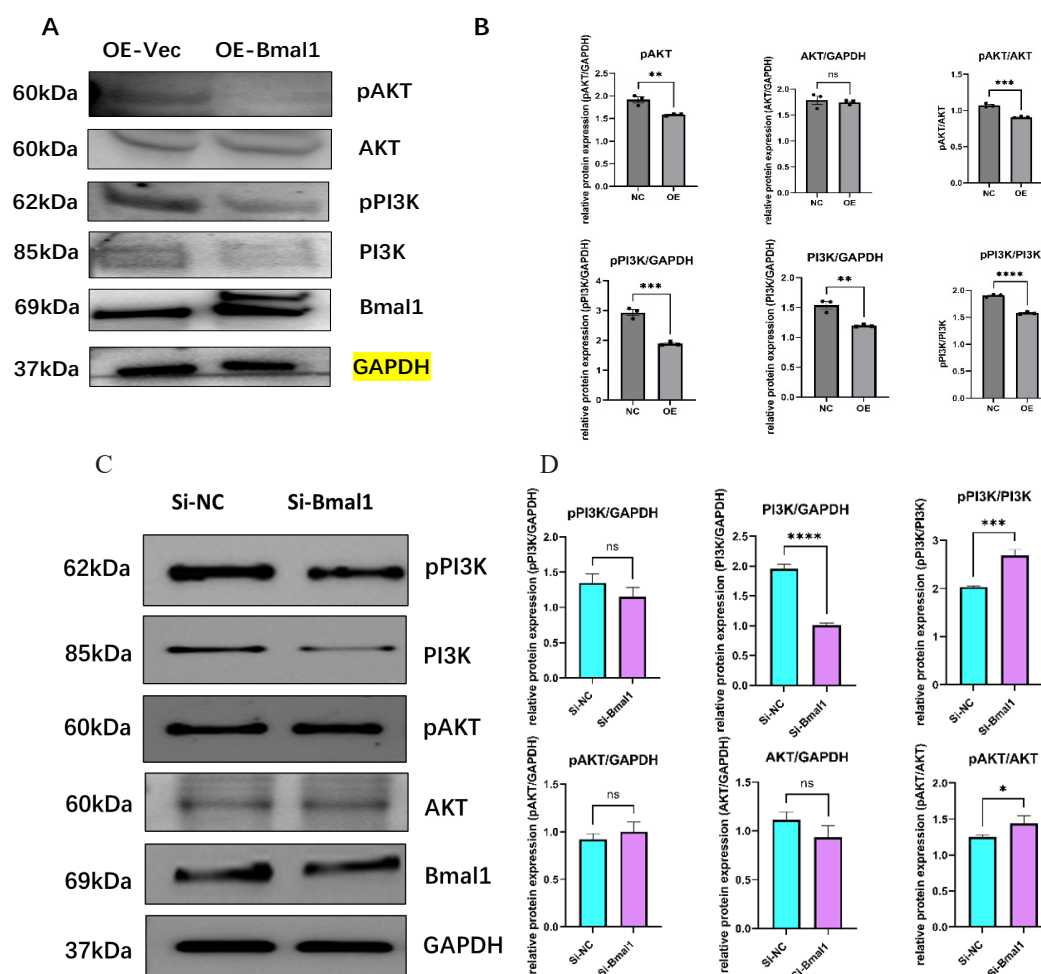


Figure 10. PI3K/Akt are differentially expressed after interfering Bmal1 in cardiomyocytes in vitro. (A) Representative western blot bands of Akt, PI3K, and brain and muscle Arnt-like protein 1 (Bmal1) in Bmal1 overexpression cardiomyocytes and control cardiomyocytes. (B) Relative expression levels of p-Akt, Akt, p-PI3K, PI3K with GAPDH used as loading control and ratios of p-Akt / Akt and p-PI3K/PI3K in Bmal1 overexpression group and control group. (C) Effect of Bmal1 knockdown on the phosphorylation of Akt and PI3K in cardiomyocytes. (D) Relative expression levels of p-Akt, Akt, p-PI3K, PI3K with GAPDH used as loading control and ratios of p-Akt / Akt and p-PI3K/PI3K in Bmal1 knock-down group and control group. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, n = 3.

2.7 Overexpression of Bmal1 reversed some of the effects of Bmal1 knockdown

To further validate the mechanism by which sleep deprivation regulates Ca^{2+} and PI3K/AKT through Bmal1, we constructed a Bmal1-overexpressing adenovirus and transfected it into neonatal mouse cardiomyocytes (Fig. 6A). The Western blot bands confirmed that Bmal1 was overexpressed in the OE-Bmal1 group (Fig. 6C) and significantly upregulated the expression of Serca2A (Fig. 7B) and NCX1 (Fig. 7C), which was a reversion of the effects Bmal1 knocking down exerted. Simultaneously, Cav1.2 ascended after Bmal1 overexpression, reversing the effect of Bmal1 knockdown in the same way. However, the level of

RyR2 was upregulated after Bmal1 overexpression and that was not consistent with the prospective results.

When Bmal1 was overexpressed in cardiomyocytes in vitro, the effects of Bmal1 downregulation on the PI3K/AKT pathway were also reversed. Through western blot bands, we observed prominent decreases in pAKT and pPI3K when comparing OE-Bmal1 group with OE-Vec group (**Fig. 8A**). Meanwhile, the phosphorylation ratios of AKT and PI3K were also significantly diminished (**Fig. 8A, B**).

These findings provide more evidence that Bmal1 may be a key regulator of SD-derived Ca^{2+} handling protein alterations and PI3K/AKT activation.

2.8 Sleep deprivation or Bmal1 interference did not make a difference in the level of Cx40

Additionally, the expression of total Cx40, which is one of the major connexins in atrial myocardium responsible for electrical conduction, did not significantly differ between the atria of acute sleep deprivation mice and mice in control group (**Fig.9A, B**). We further compared the level of Cx40 in Bmal1 overexpression group and OE-Vec group, and found no significant difference either (**Fig.9C, D**). These results collectively indicate that sleep deprivation and Bmal1 alteration may not increase the incidence of atrial fibrillation by affecting the level of Cx40.

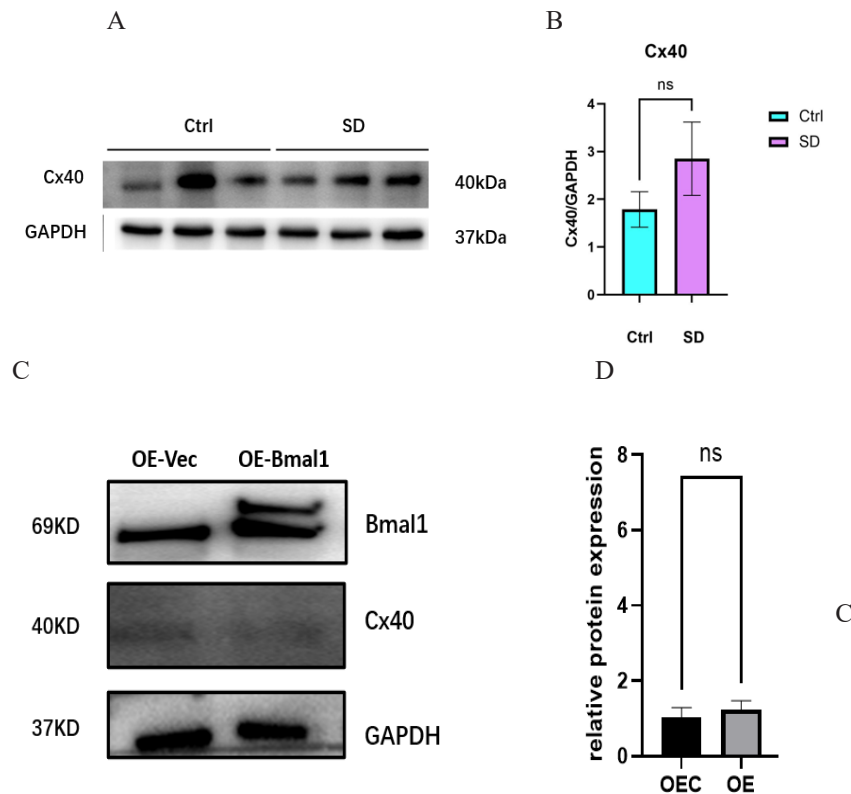


Figure 11. The influence of sleep deprivation (A, B) and Bmal1 overexpression (C, D) on Cx40 protein.

3. Discussion

In the present study, we demonstrate that sleep deprivation significantly downregulates the expression of the core circadian clock gene *Bmal1* in the murine atrium. This suppression of *Bmal1* orchestrates a pro-arrhythmic substrate through two interconnected mechanistic pathways: firstly, by inducing dysregulation of key calcium-handling proteins, characterized by a significant decrease in NCX1 and concurrent increases in RyR2 and SERCA2a, a combination predicted to promote intracellular calcium overload; and secondly, by activating the PI3K-Akt signaling pathway, as evidenced by enhanced phosphorylation of both kinases. Crucially, our *in vitro* gain- and loss-of-function studies in cardiomyocytes established a direct causal relationship, confirming that *Bmal1* knockdown recapitulates the pathological molecular signature observed in sleep-deprived mice, while its overexpression exerts protective effects. Collectively, these findings delineate a novel pathogenic axis wherein sleep disturbance impairs atrial electrophysiological and structural stability by suppressing *Bmal1*, thereby identifying *Bmal1* as a pivotal molecular mediator linking sleep disorders to increased AF susceptibility.

Of note, disturbances in circadian rhythms, particularly sleep disruption, have been increasingly recognized as a significant risk factor for atrial fibrillation (AF). Previous investigations using canine models have established that sleep disorders can increase AF susceptibility, a phenomenon linked to the downregulation of L-type calcium channels. However, a critical question remains: is this effect mediated through the perturbation of the core circadian clock component, *Bmal1*? Our study directly addresses this knowledge gap by elucidating a novel pathway wherein *Bmal1* governs the expression of key intracellular calcium-handling proteins. We demonstrated that sleep deprivation in mice led to a significant reduction in atrial *Bmal1* levels. This decrease was associated with a distinct pattern of calcium regulatory protein dysregulation: reduced expression of the sodium-calcium exchanger (NCX1) and increased levels of both sarcoplasmic/endoplasmic reticulum calcium ATPase (Serca2a) and the ryanodine receptor (RyR2). Crucially, we established a direct causal relationship in isolated cardiomyocytes. Specific knockdown of *Bmal1* faithfully replicated the sleep deprivation phenotype, confirming its central role. Conversely, *Bmal1* overexpression produced the opposite molecular signature, elevating NCX1 while suppressing Serca2a and RyR2. Collectively, these findings delineate a mechanistic cascade: sleep disturbance likely impairs atrial calcium homeostasis by suppressing *Bmal1*, which in turn alters the expression of NCX1, Serca2a, and RyR2. This dysregulation promotes intracellular calcium overload, a well-known pro-arrhythmic substrate, thereby increasing AF vulnerability. Consequently, *Bmal1* emerges as a promising potential prognostic marker for sleep disorder-related AF. However, its translation into clinical practice requires further rigorous validation through large-scale human trials to fully ascertain its diagnostic and therapeutic value.

Emerging evidence has implicated the PI3K/AKT signaling pathway in cardiac pathophysiology, including atrial fibrillation. Intriguingly, prior research has demonstrated that this pathway is significantly activated in cardiomyocyte-specific *Bmal1* conditional knockout (cKO) mice, suggesting a potential repressive role of the core circadian clock protein BMAL1 on PI3K/AKT signaling under physiological conditions. However, a critical question remains: whether pathological conditions such as sleep disturbances, known to disrupt circadian rhythms and reduce BMAL1 expression, could similarly modulate this pathway via a BMAL1-dependent mechanism. Our present study directly addresses this knowledge gap. We provide compelling evidence that sleep deprivation, a common form of sleep disorder, leads to a significant downregulation of atrial *Bmal1*, which is concurrently associated with increased phosphorylation of both PI3K and AKT. To solidify the causal link, we employed an *in vitro* approach using isolated cardiomyocytes. Consistent with our *in vivo* observations, specific knockdown of *Bmal1* robustly increased the phosphorylation levels of PI3K and AKT. Conversely, overexpression of *Bmal1* produced the opposite effect, effectively suppressing the phosphorylation of these key signaling molecules. Collectively, these data from both *in vivo* and *in vitro* models strongly suggest that sleep disturbances may enhance the phosphorylation of the PI3K-AKT axis, at least partially, through the suppression of *Bmal1*. This enhanced signaling could potentially contribute to the electrophysiological and structural remodeling that predisposes the atria to fibrillation. While these findings illuminate a novel mechanistic pathway linking sleep deprivation to AF susceptibility, the translational potential and broader clinical applicability of targeting this axis for AF management in patients with sleep disorders warrant further rigorous investigation in future clinical trials.

Although our findings highlight BMAL1 as a novel regulator of the Ca^{2+} regulating proteins NCX1, RyR2, and Serca2A channels, as well as the phosphorylation of PI3K-AKT pathway, it remains unclear how the relationship between the transformation of Ca^{2+} handling proteins and the phosphorylation of PI3K-AKT is. Additionally, when evaluating the functional impacts of the BMAL1 regulations, only *in vitro* cardiomyocytes were investigated. Further studies on the whole hearts *in vivo* in *Bmal1* knockdown mice are needed to demonstrate the impacts of low BMAL1 levels on the Ca^{2+} regulatory proteins, PI3K-AKT pathway, and the inducement rates of AF.

4 Materials and Methods

4.1. GEO data sourcing and Identification of DEGs

The GEO database (<http://www.ncbi.nlm.nih.gov/geo/>) provided all of the gene expression datasets used in this study. Two human datasets (GSE79768 for AF and GSE98566 for SD) were selected for this study. These datasets

were profiled using microarrays of atrial samples for AF and blood samples for SD. GSE79768 included atrial tissue from participants with AF and SR. In GSE79768, 13 patients receiving valvular surgery were included (7 with AF and 6 with SR). GSE98566 included gene expression data for patients with or without sleep deprivation. The GSE98566 dataset contained 11 healthy human adult subjects after 62 hours of sleep deprivation and 6 healthy control subjects with normal sleep time.

By using the GEO2R software (<http://www.ncbi.nlm.nih.gov/geo/geo2r>), a tool provided by the GEO database that relies on the R package “limma”, AF-related DEGs were screened from the GSE79768 dataset, and SD-related DEGs were obtained from the GSE98566 dataset. Genes with $p < 0.05$ and $|\log_2 \text{FC}| > 0.5$ were considered DEGs. Overlapping DEGs were extracted and visualized for further analysis using the Jvenn tool.

4.2. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis

GO integrates the world’s largest source of information about gene functions and serves as the basis for large-scale molecular biology and genetic experimental computational analysis in biomedical research. It describes the biological functions in the following three parts: CC, BP, and MF. To understand the function of DEGs in AF patients and participants with sleep deprivation, GO analysis of the overlapping genes of the co-DEGs was performed. The KEGG database has powerful graphical functions that help researchers understand metabolic pathways, and the DAVID database (<https://david.ncicrf.gov/>) offers a systematic set of functional annotation tools. Hence, we performed a KEGG pathway enrichment analysis to predict the potential functions of the robust common genes. The enriched terms with $P < 0.05$ were considered statistically significant. Both GO and KEGG pathway enrichment analysis were performed on Weishengxin platform (<https://www.bioinformatics.com.cn/>).

4.3 Animals

For sleep deprivation experiment, male C57BL/6J mice aged 6–8 weeks weighing 18–22g were purchased from Sibeifu (Beijing) Biotechnology Co., Ltd. All animals were housed in cages at room temperature ($25 \pm 1^\circ\text{C}$) with free access to food and water and were acclimatized for 7 days before the experiments. The humidity was maintained at $55 \pm 5\%$. Animal experiments were performed under a project license (No. 2023-X19-64) granted by ethics board of Chinese PLA General Hospital Medical Laboratory Animal Center, in compliance with institutional guidelines for the care and use of animals.

4.4 Induction of the acute REMSD model by MMPM

Male C57BL/6J mice, aged 6–8 weeks and weighing 18–22 g, were randomly divided into the REMSD group and the control group. A modified mul-

ti-platform water environment method (Cao et al., 2021) was used for 7 consecutive days of rapid eye movement sleep deprivation (REMSD) modeling. For the SD group, mice were placed in a water tank with the following dimensions: 44 cm (length) × 33 cm (width) × 16.7 cm (height) (Figure 1A). The water tank contained 15 circular platforms arranged in three rows. Each platform was 3 cm in diameter, separated from the other platforms in its row by 5 cm on each side, and fixed to the bottom of the tank. The water level in the tank was maintained 1 cm below the platform. This experimental design allowed the mice to move around inside the tank by jumping from one platform to another during their waking hours. The idea behind the experimental design was that the rats would fall off their platforms during REM sleep due to muscle atonia and would wake up upon contact with the water, resulting in the deprivation of REM sleep. After adaptive feeding for a week, the mice were placed in the modified multiple platform water tank for 3 hours each day, for 3 consecutive days, for adaptation before formal sleep deprivation. Then, the mice were placed on the platforms for 20 hours daily, taken out, dried, and placed back in clean and dry cages for 4 hours for rest (17:00-21:00). This cycle was repeated seven times. For the control group, mice were placed in normal cages. During the modeling, the mice had ad libitum access to food and daily-changed water. In each group, mice were sacrificed immediately after sleep deprivation ended, and their heart tissues were collected for further experiments.

4.5 Hematoxylin and eosin (H&E) staining and Masson trichrome staining

Mice were deeply anesthetized with an intraperitoneal injection of 0.1ml/10 g of chloral hydrate, after which heart tissues were harvested and fixed in 4% paraformaldehyde. The hearts were embedded in paraffin and sectioned at 4 μm intervals. The microstructure of the heart was assessed using hematoxylin–eosin (H&E) staining, and the distribution of collagen fibers in cardiac tissues was evaluated using Masson staining. The changes were analyzed under a light microscope and photographed using Case Viewer software. In Masson staining, the blue color in tissues indicated the presence of collagen fibers. After the completion of staining, Image J software was used to calculate the proportion of collagen fibers: collagen fiber ratio=collagen fiber area/myocardial cross-sectional area. Six mice (3 mice in SD group and 3 mice in control group) were sacrificed respectively in H&E and Masson staining.

4.6 Neonatal Mice Cardiomyocyte Culture and Adenovirus Transfection

Cardiomyocytes were isolated from 1- to 3-day-old C57BL/6J mice hearts as described previously (Wang et al. 2012; Wang et al. 2009; Wang et al. 2015). The cardiomyocyte culture medium was Dulbecco's Modified Eagle Medium (DMEM) supplemented with 4.5g/L D-Glucose, L-Glutamine, 110mg/L Sodium

Pyruvate, 20% Gibco fetal bovine serum, glutamine 1%, penicillin 1% and streptomycin 1%. The cardiomyocytes were cultured at 37°C under 95% oxygen and 5% CO₂.

The recombinant adenoviruses HBAD-m-Brnt1-3×flag-EGFP and HBAD-Adeasy-m-Bmal1 shRNA1-EGFP were purchased from Hanbio (Shanghai, China), labeled with GFP. The adenoviruses HBAD-EGFP overexpression control and HBAD-EGFP NC control were used as negative controls. The multiplicity of adenovirus infection (MOI) was 30. GFP was detected under a fluorescence microscope 48 hours after the transfection. In the experiments, the cardiomyocytes were plated in the 6-well plates (3×10⁵ cells/well) after isolation, and were cultured at 37°C under 95% oxygen and 5% CO₂. The fibroblasts were removed by the differential adhesion method and by using 5-bromo-2'-deoxyuridine (BrdU). After 48 hours, the cardiomyocytes were transfected with adenoviruses utilizing 1/2 small volume infection method. The culture medium was replaced with half the volume of fresh medium containing the recombinant adenoviruses, in which the cardiomyocytes were incubated for 4 hours. Then, another half of the volume of fresh medium was added. After the transfections were completed (10–16 hours after transfection), the cardiomyocytes were cultured in fresh medium for 48 hours before the Western blotting experiments were performed. The experiments were repeated three times.

4.7 Western Blot Analysis of Protein Levels in the Atria and Cardiomyocytes

Mouse atrium samples or cardiomyocytes were ground in liquid nitrogen, homogenized in lysis buffer (RIPA lysis buffer, 1% PMSF, and 1% phosphatase inhibitor). Protein fractions were collected by centrifugation at 12,000 rpm at 4 °C for 30 min. The protein concentration in the supernatant was measured by a BCA assay kit. Equal amounts of protein samples were loaded on 4% - 20% SDS polyacrylamide gels and transferred to PVDF membranes. The membranes were blocked with 5% skimmed milk or 5% BSA blocking buffer (for phosphorylated proteins) for 2 hours at room temperature and then rinsed three times with TBST (TBS containing 0.1% Tween-20) 3 minutes for 3 times. Then, the membranes were incubated with primary antibodies at 4°C overnight (18–24 hours). After 2 hours of incubation at 37°C with horseradish peroxidase-conjugated secondary antibodies, the membranes were rinsed 10 minutes for 3 times with TBST. (This sentence is actually fine grammatically, though it seems like a fragment. A more complete sentence might be: “I performed the experiment for 3 times with TBST. The protein bands were visualized using the ECL luminescent developer. Images were captured using a Tanon 5200 imaging system.

Primary antibodies against Bmal1 (1:1000, A4714, ABclonal) , RyR2 (1:1000, ab302716, Abcam), SERCA2a (1:1000, A11692, ABclonal), Cav1.2 (1:1000, 21774-1-AP, Proteintech), NCX1 (1:5000, ab177952, Abcam), Akt total (1:2000, A18675, ABclonal), p-AktSer473 (1:1000 ,AP0637, ABclonal), PI3K

p85(1:9000, A22996, ABclonal), p-PI3K(1:5000, AP1463, ABclonal) , Cx40 (1:1000, A7231, ABclonal) , GAPDH (1:5000, HX1828-100, Huaxingbio) were used, and secondary antibodies used were HRP-goat anti-rabbit IgG (1:5000, AS014, ABclonal) and HRP-goat anti-mouse IgG (1:5000, AS003, ABclonal).

All protein aliquots were stored at -80°C until use, with three independent biological replicates performed for each experimental condition.

4.8 RNA Extraction and Real-Time Quantitative PCR

Total RNA from the mouse atrium was extracted using TRIzol. The RT-qPCR was performed as previously described. After normalizing with GAPDH, the relative levels of target RNAs were calculated using the $\Delta\Delta\text{CT}$ method. The primer sequences are as follows: Bmal1: sense: 5-ATAAGGACTTCGCCTC-TACCTGTTCA-3, Antisense: 5-CTCGTTGTCTGGCTCATTGTCTT -3.

4.9 Data and Statistical Analysis

All experiments were repeated three times. Results were expressed as the mean $\pm$ standard deviation. The difference between groups was analyzed using the Student's t-test and Mann-Whitney U test, employing GraphPad Prism version 9.5 software. A P-value < 0.05 was considered significantly different.

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