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Camk2a suppresses denervated muscle atrophy by maintaining the Ca^{2+} homeostasis in muscle cells

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ABSTRACT

Denervated muscle atrophy is a severe neurological complication that significantly impacts patients' quality of life. Currently, there is a lack of effective treatment methods. This study aims to investigate the molecular mechanisms associated with denervated muscle atrophy and explore potential therapeutic targets. In this study, we assessed the severity of denervated muscle atrophy by measuring the wet-weight ratio of the calf muscles. We conducted Western blot and immunofluorescence experiments to observe the morphology and cross-sectional area of muscle fibers following sciatic nerve transection. Simultaneously, we evaluated the expression of Camk2a in muscle tissue and measured changes in Ca^{2+} using the BCA method. Additionally, we performed HE and Sirius Red staining on denervated muscle tissue to observe the cross-sectional area of muscle fibers and collagen deposition in response to Camk2a overexpression. In our study, We observed a significant decrease in the wet weight ratio of the muscles, myosin, and muscle fiber cross-sectional area with the prolonged duration of sciatic nerve transection. Subsequently, we observed varying degrees of elevation in Ca^{2+} levels in denervated muscle tissue, while Camk2a, which regulates Ca^{2+} signal transduction, significantly decreased in denervated muscle tissue. Overexpression of Camk2a reduced the accumulation of Ca^{2+} in muscle tissue, resulting in higher muscle wet weight ratios, larger muscle fiber cross-sectional areas, and a significant reduction in collagen deposition in muscle tissue. In conclusion, our study provides the first evidence that Camk2a can alleviate calcium overload in muscle cells and ameliorate denervated muscle atrophy. Our findings suggest that Camk2a may serve as a crucial regulatory target in denervated muscle atrophy.

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Introduction

The neural control of skeletal muscle fibers plays a crucial role in maintaining muscle tone and normal functioning (1). In various pathological conditions such as trauma, diabetic neuropathy, degenerative disc disease, alcoholic neuropathy, amyotrophic lateral sclerosis (ALS), spinal muscular atrophy, peroneal muscular atrophy, and spinal muscular atrophy, prolonged loss of neural control over muscle fibers results in a decrease in muscle mass and strength, a condition known as denervated muscle atrophy (2, 3). While both domestic and international scholars have conducted extensive theoretical and clinical research on the underlying diseases mentioned above, the recovery of neural function is a very slow process. Prolonged loss of neural stimulation to muscles can lead to irreversible muscle atrophy, significantly impacting the quality of life for patients. Therefore, understanding the pathogenic mechanisms of denervated muscle atrophy is of paramount importance in actively preventing muscle atrophy while treating the underlying diseases mentioned above.

Skeletal muscles are rich in mitochondria, which are essential for maintaining their normal functions. The most significant pathological change during denervated muscle atrophy is the imbalance between protein synthesis and degradation within muscle tissues, with mitochondrial dysfunction playing a crucial role in this process (4).

Mitochondrial dysfunction leads to the production of a large amount of reactive oxygen species (ROS), resulting in increased oxidative protein content within muscle cells. This further activates the ubiquitin-proteasome system and accelerates protein degradation (5, 6). Additionally, increased permeability of the inner and outer mitochondrial membranes promotes the release of cytochrome c between these membranes, inducing apoptosis in muscle cells and exacerbating the progression of muscle atrophy (7). Recent research has discovered the importance of calcium (Ca^{2+}) balance in mitochondria for muscle tissue function. During muscle contraction, the influx of Ca^{2+} into mitochondria can activate key enzymes in the oxidative respiratory chain, facilitating ATP production and providing energy for muscle activity. In cases of muscle atrophy, mitochondrial Ca^{2+} overload disrupts the permeability of mitochondrial membranes, leading to the production of ROS and the release of cytochrome c, thus playing a significant role in the occurrence of muscle atrophy (8).

Camk2a, a member of the Ca^{2+}/calmodulin-dependent protein kinase (Camk) family, is a multifunctional serine/threonine protein kinase that plays a crucial role in Ca^{2+} signal transduction (9, 10). However, its role in denerva-

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ted muscle atrophy is not yet clear. In this study, we have, for the first time, reported the significant role of Camk2a in denervated muscle atrophy. In a mouse model of lower limb denervated muscle atrophy following sciatic nerve transection, we observed a notable increase in Ca^{2+} levels, accompanied by a significant downregulation of Camk2a expression. By promoting the expression of Camk2a in mouse muscle tissues using adenoviruses, we were able to achieve higher muscle wet weight and larger muscle fiber cross-sectional areas, thus delaying the progression of denervated muscle atrophy in mice. Our research indicates that Camk2a may represent an important target for the treatment of denervated muscle atrophy.

Materials and Methods

Establishment of a denervated muscle atrophy model

C57BL/6J male mice, aged 8-10 weeks, were sourced from Jiangsu Huachuang Sino Medical Technology Co., Ltd (Taizhou, China). In our study, all mice had ad libitum access to food and water and were kept on a 12-hour light-dark cycle. In the experiment, the right sciatic nerve of the mice was transected, while the control group underwent only a skin incision, blunt dissection of the muscle, and exposure of the sciatic nerve without further treatment. The surgical procedure was conducted as described by Xiang et al. (11). The experimental mice were randomly divided into three groups: the Sham group (control), the denervation group, and the denervation + AAV-Camk2a group. Four weeks before the surgery, adenovirus overexpressing Camk2a (AAV-Camk2a) was injected into the mice's calf muscles, and the overexpression effect was verified using PCR. After surgery, at specific time points, the mice were anesthetized and euthanized, and their calf muscles were dissected for further experiments.

Muscle Wet Weight Ratio, Hematoxylin and Eosin (HE) Staining, and Sirius Red Staining

At 1, 2, 3, and 4 weeks after the operation, the mice were euthanized following anesthesia, and the calf muscles including extensor digitorum longus (EDL), soleus (SOL), and gastrocnemius (GAS) were isolated and weighed. Muscle atrophy was assessed by calculating the ratio of the mass of the affected side to the healthy side (wet weight ratio). For HE and Sirius Red staining, the procedure involved fixing the dissected mouse gastrocnemius muscle in formaldehyde for 24 hours. Subsequently, the muscle tissue was embedded in paraffin and sectioned. Following deparaffinization and hydration, muscle tissue staining was performed using HE (C0105S, Beyotime Biotechnology, Shanghai, China) and Sirius Red staining (G1472, Solarbio, Beijing, China) kits according to the provided instructions. After completing the staining process, the specimens were observed under an optical microscope.

Immunofluorescence

After obtaining paraffin sections of mouse muscle tissue and conducting deparaffinization and heat antigen retrieval, the sections were incubated at room temperature with an endogenous peroxidase-blocking solution (P0100B, Beyotime, Shanghai, China) for 10 minutes. Subsequently, block the sections with an immunofluorescence-blocking solution at room temperature for one hour. Then, incubate the sections with Camk2a (1:100, 54477, SAB, Nanjing, China) and Laminin (1:100, ab11575, Abcam, Cambridge, MA, USA) specific antibody overnight at 4°C. On the following day, incubate the sections with a specific fluorescent secondary antibody at room temperature in the dark for 1 hour. After nuclear counterstaining, use a fluorescence microscope to observe Camk2a /Laminin expression in the muscle tissues.

Western blotting

The fresh mouse muscles were crushed and then added to a protein lysis buffer (KGP2100, KeyGEN, Nanjing, China). Utilize ultrasonication for further tissue breakdown. After centrifugation, collect the supernatant. Measure the protein concentration of a portion of the supernatant using a bicinchoninic acid (BCA) assay kit (P0010, Beyotime Biotechnology, Shanghai, China), while the remaining portion should be boiled for subsequent experiments. For the gel electrophoresis experiments, add an equal amount of tissue protein from each group. Use a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel for protein separation. Subsequently, transfer the proteins to a polyvinylidene fluoride (PVDF) membrane. After a 15-minute incubation in rapid blocking solution, add the specific primary antibodies as follows: Anti-MHC (1:1000, MF20, R&D Systems, Minneapolis, MN, USA), Anti-Camk2α (1:1000, 54477, SAB, Nanjing, China), GAPDH (1:10,000, HRP-60004, Proteintech, Rosemont, IL, USA). Incubate at 4°C overnight. On the following day, apply specific secondary antibodies and incubate at room temperature for one hour. Use a chemiluminescence system (4600; Tanon Science and Technology Co., Ltd., Shanghai, China) for detection and analysis of the results with Ima J software.

RT-qPCR

After extracting mouse muscle tissue, RNA was extracted from the tissue using an RNA total extraction kit (Yi Fei Xue Biotechnology, Nanjing, China). Subsequently, the RNA content in the mouse muscle tissue was measured using a NanoDrop 2000 spectrophotometer. The RNA was reverse-transcribed into cDNA using an RNA reverse transcription kit (Yi Fei Xue Biotechnology, Nanjing, China), 1× SYBR was added, and real-time fluorescent quantitative PCR was performed using a Roche 480 instrument for detection (Roche, Basel, Switzerland). The primer information used in the experiment is as follows: GAPDH: Forward Primer: AGGTCGGTGTGAACGGATTTG, Reverse Primer: GGGGTCGTTGATGGCAACA; Camk2α: Forward Primer: TGGAGACTTT GAGTCCTACACG; Reverse Primer: CCGGGACCACAGGTTTTCA.

Quantification of Ca^{2+} Concentration Using BCA Method

The procedure involves testing the Ca^{2+} (calcium ion) content within muscle tissue using a calcium content colorimetric assay kit (S1063S, Beyotime Biotechnology, Shanghai, China). In a simplified description, the tissue is first cut into small pieces, added to an appropriate sample solubilization solution, thoroughly homogenized using a grinder, and then centrifuged to extract the supernatant. In a 96-well plate, 50 μL of standard or sample is added to each well, followed by the addition of 150 μL of the assay reagent. After incubation at room temperature in the dark for 10 minutes, absorbance is measured using a microplate

reader, and the Ca^{2+} content within the tissue is calculated.

Statistical Analysis

In this experiment, all quantitative data are presented as mean ± standard deviation. Subsequently, statistical analyses were performed using Prism software. Statistical methods included unpaired t-tests, one-way or two-way analysis of variance (ANOVA), followed by Tukey post hoc tests. An assumed value of $P < 0.05$ was considered statistically significant.

Results

Establishment of the Denervated Muscle Atrophy Model

We first compared the degree of muscle atrophy by dissecting the muscles of the posterior calf group in denervated mice at different time points and observing the muscle mass compared to the contralateral healthy side. Similar to the results of Yang et al. (12), regardless of whether it was the gastrocnemius, extensor digitorum longus, or soleus muscle wet weight ratio, there was a varying degree of decrease with increasing denervation time (Figure 1A). Subsequently, we further examined the protein expression of myosin-heavy chains in muscle tissues. Similar to the wet weight ratio results, myosin heavy chain expression gradually decreased in all posterior calf muscle tissues with increasing denervation time (Figure 1B), and this difference was statistically significant compared to the control group (Figure 1C). To further observe the morphological changes in denervated muscles, we stained muscle fibers with Laminin. The study results indicated that with the passage of time, both the diameter and area of muscle fibers gradually decreased, suggesting the establishment of a denervated muscle atrophy model.

Mitochondrial Dysfunction and Marked Decrease in Camk2a Expression in Denervated Muscle Tissue

In order to observe the ultrastructural changes in denervated muscle tissues, we conducted scanning electron microscopy (SEM) analysis. The results revealed that, after 4 weeks of denervation, the myofibers of the mouse GAS muscle were severely damaged compared to the control group. This damage was characterized by a significant disarray in myofiber alignment, twisted and fragmented Z-lines, along with mitochondrial swelling and vacuolization, and a reduction in the number of mitochondria (Figure 2A-B). Subsequently, we measured the intracellular Ca^{2+} content using a Ca^{2+} assay kit. The results showed a significant increase in calcium ions within denervated muscle tissues (Figure 2C). Next, we assessed the expression of the important calcium signaling regulator protein Camk2a. Western Blot results revealed a significant decrease in Camk2a expression in the mouse gastrocnemius muscle after 4 weeks of denervation (Figure 2E). This was further confirmed by tissue fluorescence experiments (Figure 2D). These results suggest that Camk2a may be a critical target in regulating muscle atrophy.

Overexpression of Camk2a Alleviates the Progression of Denervated Muscle Atrophy.

In light of the research findings presented above, in order to gain further insight into the crucial role of Camk2a in the process of muscle atrophy, we conducted experiments involving the injection of adenoviruses that overexpress Camk2a into mouse muscle tissue. This was done to enhance Camk2a expression in the gastrocnemius muscle of the mice. PCR results indicated a significant increase in Camk2a expression in the overexpression group compared to the denervated group (Figure 3A). Additionally, the analysis of calcium ion (Ca^{2+}) levels in mouse muscle tissue demonstrated a significant decrease in Ca^{2+} content in the overexpression group compared to the denervated group (Figure 3B). Moreover, the overexpression group exhibited a higher gastrocnemius muscle wet weight ratio (Figure 3C). Subsequently, we conducted histological analyses using Hematoxylin and Eosin (HE) staining and Sirius Red staining to further examine the diameter of muscle fibers and the deposition of collagen fibers in denervated muscle tissue (Figure 3D). The quantitative results show that, compared to the control group, the denervated group exhibited a significant reduction in muscle fiber diameter and an increase in collagen deposition. Overexpression of

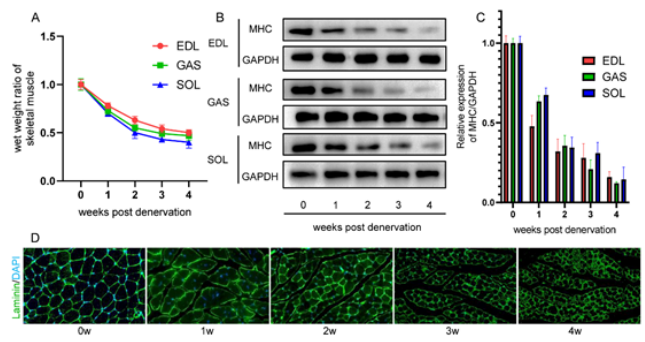

Figure 1. Establishment of the Denervated Muscle Atrophy Model. (A) The wet weight ratio of the EDL, GAS, and SOL muscles at different time points after sciatic nerve transection. (B-C) Detection of myosin heavy chain (MHC) expression in the EDL, GAS, and SOL muscles at different time points after sciatic nerve transection using Western blot, with GAPDH as an internal reference. (D) Observation of muscle fiber size in the gastrocnemius muscle through Laminin staining (Laminin: green, DAPI: Blue), the magnification is 400x.

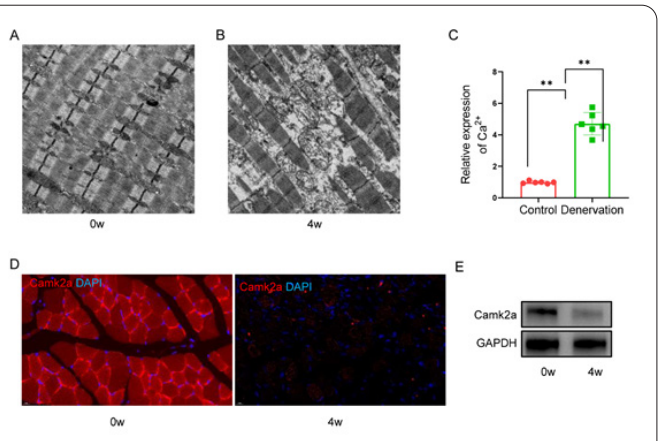

Figure 2. Mitochondrial Dysfunction and Marked Decrease in Camk2a Expression in Denervated Muscle Tissue. (A-B) Scanning electron microscopy for observing gastrocnemius muscle fiber and mitochondrial morphology. (C) Ca^{2+} expression levels in each group. (D-E) Detection of Camk2a expression in gastrocnemius muscle tissue using immunofluorescence (Camk2a: Red, DAPI: Blue), the magnification is 400× and Western Blot methods (GAPDH as internal reference). Data were presented as mean ± SD. n = 6. *P < 0.05, **P< 0.01.

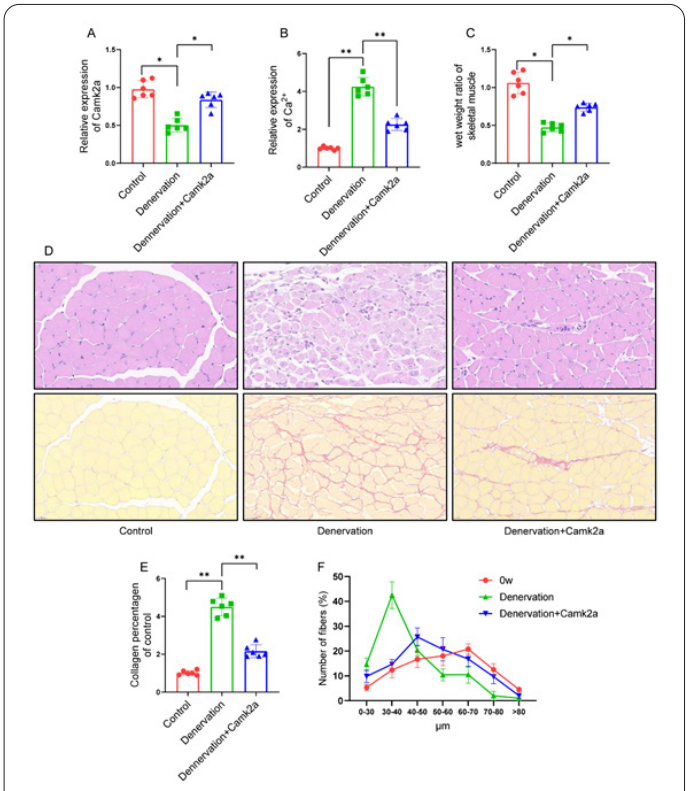

Figure 3. Overexpression of Camk2a alleviates denervated muscle atrophy. (A) Camk2a expression levels in normal muscle, atrophic muscle, and after Camk2a treatment. (B) Ca2+ expression levels in each group. (C) Wet weight ratio (surgical side weight divided by contralateral weight) of the gastrocnemius (GAS) muscle at specified time points in normal, denervated, and treated groups. (D) Observation of gastrocnemius muscle morphology using H&E staining and Sirius Red staining in different groups, the magnification is 400×. (E) Quantification of collagen deposition area in each group. (F) Quantification of muscle fiber diameter in each group. Data were presented as mean ± SD. n=6, *P<0.05, **P<0.01.

Camk2a significantly improved the aforementioned conditions (Figure 3E-F). The research findings outlined above collectively indicate that overexpression of Camk2a significantly delays the onset of denervated muscle atrophy.

Discussion

The atrophy of denervated muscles is a complication of nerve injuries that significantly affects the patient's quality of life. Therefore, understanding the pathogenic mechanisms of denervated muscle atrophy is of utmost importance. In this study, we explored the pivotal role of Camk2a in the pathogenesis of denervated muscle atrophy, providing a novel insight into potential therapeutic targets for this condition.

First, we observed mitochondrial dysfunction and excessive accumulation of intracellular Ca^{2+} in muscle tissues of a denervated muscle atrophy mouse model (Figure 2), consistent with findings from previous studies by Csukly et al. (13). Calcium, the fifth most abundant element in the human body and the most abundant metal element, is essential for various biological functions (14-16). Calcium metabolism involves maintaining calcium balance and calcium homeostasis. Calcium balance refers to the equilibrium in the body's calcium storage, primarily related to the state of the bone calcium pool. Calcium homeostasis pertains to the stable regulation of ionized calcium levels in the intracellular and extracellular fluids. Under normal circumstances, intracellular Ca^{2+} concentration is precisely regulated to maintain its stable level, supporting normal cellular activities. However, excessive intracellular Ca^{2+} accumulation can lead to mitochondrial dysfunction and further activation of the ubiquitin-proteasome system, promoting protein degradation and accelerating muscle atrophy (17, 18). Therefore, inhibiting calcium overload in denervated muscle tissues and restoring Ca^{2+} homeostasis may be a critical research direction in mitigating muscle atrophy.

Camk2 is a multifunctional serine/threonine protein kinase and a crucial factor in calcium signal transduction, regulating various biological functions in cells. These functions include amino acid and lipid metabolism, synthesis and release of neurotransmitters, and maintenance of intracellular calcium homeostasis, among others. Camk2a is a specific subtype primarily expressed in neural tissues (19-21). Studies have shown that regulating Camk2a expression in neural tissues, such as the brain and spinal cord, enhances various cognitive abilities and delays the progression of conditions like Alzheimer's disease and depression (22, 23). In this study, we found a significant increase in Camk2a expression in denervated muscle tissues, which effectively mitigated Ca^{2+} deposition and delayed the progression of muscle atrophy (Figure 3). To our knowledge, this is not the first report of Camk2a's role outside neurological diseases. For example, Park H and colleagues discovered that modulating the Ca^{2+}-Camk2a-CREB1 pathway can promote extracellular matrix mineralization in osteoblasts (24). Furthermore, Camk2a plays a crucial role in cancer-related diseases, inhibiting the progression of tumors in lung cancer, breast cancer, and glioblastoma, thereby improving patient prognosis (25-27).

While we successfully demonstrated that adenovirus-mediated overexpression of Camk2a in denervated muscle tissues inhibits muscle atrophy, we did not explore the specific mechanisms through which Camk2a exerts its effects in denervated muscle atrophy. Thus, future studies will delve into conducting experiments to investigate the precise mechanisms of Camk2a's role in this context. Additionally, one limitation of this study is the relatively narrow range of experimental methods employed. Expanding the research methodology in future work to validate our findings from various perspectives will be highly beneficial.

In conclusion, our study provides the first evidence that Camk2a can alleviate calcium overload in muscle cells and ameliorate denervated muscle atrophy. Our findings suggest that Camk2a may serve as a crucial regulatory target in denervated muscle atrophy.

Ethical compliance

This study was approved by the Animal Ethics Committee of Nanjing Medical University Animal Center

Conflict of interest

The authors have no potential conflicts of interest to report relevant to this article.

Author contributions

LY designed the experiments. YZ and MX performed the animal experiments. TZ, QZ and RL analyzed the data. YZ wrote the paper. LY funded and supervised the research. All authors read and approved the final manuscript.

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