α-Synuclein aggregation causes muscle atrophy through neuromuscular junction degeneration

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Abstract

Background   Sarcopenia is common in patients with Parkinson's disease (PD), showing mitochondrial oxidative stress in skeletal muscle. The aggregation of α-synuclein (α-Syn) to induce oxidative stress is a key pathogenic process of PD; nevertheless, we know little about its potential role in regulating peripheral nerves and the function of the muscles they innervate.

Methods   To investigate the role of α-Syn aggregation on neuromuscular system, we used the Thy1 promoter to overexpress human α-Syn transgenic mice (mThy1-hSNCA). hα-Syn expression was evaluated by western blot, and its localization was determined by confocal microscopy. The impact of α-Syn aggregation on the structure and function of skeletal muscle mitochondria and neuromuscular junctions (NMJs), as well as muscle mass and function were characterized by flow cytometry, transmission electron microscopy, Seahorse XF24 metabolic assay, and AAV9 in vivo injection. We assessed the regenerative effect of mitochondrial-targeted superoxide dismutase (Mito-TEMPO) after skeletal muscle injury in mThy1-hSNCA mice.

Results   Overexpressed hα-Syn protein localized in motor neuron axons and NMJs in muscle and formed aggregates. α-Syn aggregation increased the number of abnormal mitochondrial in the intramuscular axons and NMJs by over 60% ($P < 0.01$), which inhibited the release of acetylcholine (ACh) from presynaptic vesicles in NMJs ($P < 0.05$). The expression of genes associated with NMJ activity, neurotransmission and regulation of reactive oxygen species (ROS) metabolic process were significantly decreased in mThy1-hSNCA mice, resulting in ROS production elevated by ~220% ($P < 0.05$), thereby exacerbating oxidative stress. Such process altered mitochondrial spatial relationships to sarcomeric structures, decreased Z-line spacing by 36% ($P < 0.05$) and increased myofibre apoptosis by ~10% ($P < 0.05$). Overexpression of α-Syn altered the metabolic profile of muscle satellite cells (MuSCs), including basal respiratory capacity (~170% reduction) and glycolytic capacity (~150% reduction) ($P < 0.05$) and decreased cell migration and fusion during muscle regeneration (~60% and ~40%, respectively) ($P < 0.05$). We demonstrated that Mito-TEMPO treatment could restore the oxidative stress status (the complex I/V protein and enzyme activities increased ~200% and ~150%, respectively), which caused by α-Syn aggregation, and improve the ability of muscle regeneration after injury. In addition, the NMJ receptor fragmentation and ACh secretion were also improved.

Conclusions   These results reveal that the α-synuclein aggregation plays an important role in regulating acetylcholine release from neuromuscular junctions and induces intramuscular mitochondrial oxidative stress, which can provide new insights into the aetiology of muscle atrophy in patients with Parkinson's disease.

Keywords   Area; Parkinson's disease; Muscle atrophy; Oxidative stress; Neuromuscular junction; α-Synuclein

Introduction

Parkinson disease (PD) is a common neurodegenerative disease in elderly people. As the disease progresses, PD patients have different degrees of muscle weakness, muscle fatigue, muscle pain and muscle atrophy.[1] It is defined as sarcopenia when the reduction in muscle mass reaches 10–20% of that of a normal adult and is accompanied by a decrease in strength and/or physical function.[2,S1] It is associated to several negative outcomes such as falls and disability. Compared with non-PD patients, PD patients have a higher prevalence of sarcopenia and frequency of falls.[3,S2] Despite its importance, few studies have explored the exact mechanism of muscle atrophy in this population.

Muscle atrophy is mainly regulated by the balance between skeletal muscle damage and repair. Effective muscle regeneration can avoid the occurrence of muscle atrophy, which is affected by the activation, proliferation and differentiation of muscle satellite cells (MuSCs) located in the basement membrane of myofibres. During myogenesis, MuSCs will undergo extensive metabolic changes.[4,S3] Therefore, pathologies characterized by metabolic disorders may impair myogenesis, resulting in decreased muscle regeneration capacity or muscle atrophy.[4] It is clinically found that the duration, severity and subtype of PD affect myofibre niche.[5] Studies have reported that abnormal morphology of mitochondria in skeletal muscle of PD patients, as well as decreased activity of respiratory chain complex I (complex I), declined ATP production and membrane potential, suggesting that the skeletal muscle mitochondria of PD patients have impaired oxidative phosphorylation (OXPHOS) and increased peroxide production, which intensifies oxidative stress.[6] Previous studies have found that when denervation or neuromuscular junction (NMJ) degeneration, such as biological ageing, the production of peroxides in muscle mitochondria increases significantly, leading to apoptosis, and it will also eliminate the interaction between motor neurons and MuSCs, resulting in obvious defects in muscle regeneration.[7,8,S4] These disruptions are often associated with skeletal muscle dysfunction and diseases. However, whether the oxidative stress of skeletal muscle mitochondria in PD patients is related to ageing remains unclear.

The accumulation and dissemination of the pathogenic protein α-synuclein (α-Syn) in the brain is a key factor in the development of PD,[9] whereas the expression of α-Syn is not limited to the brain. It is also expressed in skeletal muscle and heart.[10,11] Studies have reported that the intramuscular motor neurons and NMJs of patients with sporadic PD dysphagia are rich in high-density α-Syn aggregates, and compared with non-dysphagia PD patients, the density of α-Syn aggregates is higher in PD patients with dysphagia.[12] The transgenic mouse models overexpressing α-Syn using a variety of different promoters all showed different degrees of peripheral neuropathy and muscle atrophy, indicating that α-Syn may be involved in regulating skeletal muscle

function.[13,14] A body of evidence has shown that α-Syn localizes to mitochondria and contributes to the disruption of key mitochondrial processes.[15,16,S5] Studies performed in SH-SY5Y cells overexpressing α-Syn A53T mutant or wild type and in isolated rat brain mitochondria showed that interaction of α-Syn with mitochondria causes oxidative modification of mitochondrial components.[17] α-Syn also regulates the function of mitochondrial translocators, electron transport chain (ETC) proteins or fusion–fission proteins by direct protein–protein interactions.[16,18] Mitochondrial accumulation of α-Syn has been associated with impaired complex I-dependent respiration, decreased mitochondrial membrane potential and increased levels of mitochondrial reactive oxygen species (ROS) in multiple cellular models.[17,19,S6] The evidence supporting the contribution of abnormal accumulation of α-Syn to disruption of mitochondrial processes is compelling and indicates a crucial role for α-Syn-induced mitochondrial oxidative stress in PD pathogenesis and α-synucleopathies. However, the role of α-Syn in PD muscle atrophy and its underlying mechanisms are poorly understood, and whether the accumulation of α-Syn leading to skeletal muscle oxidative stress is the main factor of PD muscle atrophy is still unclear.

In this study, we reported that mThy-hSNCA mice exhibited intramuscular mitochondrial oxidative stress and delayed muscle regeneration. This is mainly due to the overexpression of α-Syn, which caused mitochondrial swelling and cristae breakage in NMJs, leading to NMJ degeneration. These phenotypes can be alleviated by mitochondrial targeting of superoxide dismutase (Mito-TEMPO). These results highlight the importance of the interaction between the neural network and α-Syn during the apoptosis and regeneration of PD muscle cells and help explain the underlying mechanism of how α-Syn can cause skeletal muscle dysfunction in PD patients.

Materials and methods

Animal studies

All procedures were approved by the Animal Care and Use Committee of the Capital Medical University. mThy1-hSNCA mice were presented from Yang Hui Lab. Mice were maintained on a C57BL/6N background. 6 months (m), and 18–20 m male mice were used for this study. Unless specifically marked, all results were obtained from four to six mice for each group. For further details, see *Data S1*.

Transmission electron microscopy

Muscle tissues (1 mm³) were fixed in glutaraldehyde (2.5%), post-fixed with 1% osmium tetroxide followed by 1% uranyl

acetate, dehydrated them through a graded series of ethanol washes and embedded them in resin. The ultrathin sections stained with uranyl acetate followed by lead citrate and viewed on a JEOL 1230 EX transmission electron microscopy (TEM) at 80 kV.

MuSC isolation and fluorescence-activated cell sorting (FACS)

Normal and 1d injured tibialis anterior (TA) muscle were harvested from 6 m mice, and then they were incubated in 20 mL of DMEM media containing 0.2% collagenase-II and 2.5 U/mL dispase for 90 min at 37°C. Cell suspension was then filtered using strainer with pore size of 40 μm (BD). The mononuclear cells were suspended in PBS with 3% FBS (Hyclone) for fluorescence staining. Cells were then sorted and analysed by flow cytometry. Markers for MuSC isolation were $CD31^-CD45^-Sca\text{-}1^-CXCR4^+$. Approximately 50 000 MuSCs can be obtained from TA muscle. Data were analysed by FlowJo7.6.5. For antibody information, see *Data S1*.

Mitochondrial isolation and respiratory chain complex activity assay

100 mg TA muscles were gently crushed in 1.0 mL of ice-cold isolation buffer, then centrifuge at 600 g at 4°C for 10 min. Transfer the supernatant to another tube and centrifuge at 11 000 g at 4°C for 15 min. The isolated buffer was added to the precipitate, which was sonicated. The activities of complexes I–V were measured by respiratory chain complex kits (Solarbio).

OxyBlot assay and GSH/GSSG measurement

For detection of protein oxidation, an OxyBlot Protein Oxidation Detection Kit (Merck Millipore) was used according to the manufacturer's instructions. The band intensity was analysed using ImageJ software. GSH/GSSG ratios from 10 male mice TA muscle were measured by using a glutathione assay kit (Beyotime).

ATP measurement

The muscle homogenized in 20 mL of homogenization buffer (6 M guanidine-HCl, 100 mM Tris and 4 mM EDTA pH 7.8) was centrifuged at 16 000 g. The supernatants diluted 1:1000 and 1:10 with water were subjected to measurements of ATP and protein concentrations, respectively. ATP and protein concentrations were determined using the CellTiter-Glo Luminescent Cell Viability assay kit (Promega) and the BCA protein assay kit (Pierce), respectively.

Analysis of mitochondrial bioenergetics

Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were recorded in MuSCs using Seahorse XF24e analyser (XF-Cell-Mito-Stress Test Kit). XF-Cell-Glycolysis-Stress Test Kit (Seahorse Biosciences) was used for glycolytic rate analysis. For further details, see *Data S1*.

ROS determination

For determination of ROS production in muscle sections, frozen TA muscle cross sections were washed with ice-cold PBS for 5 min, incubated with 300 nM $CM\text{-}H_2DCFDA$ (Invitrogen) in PBS at 37°C for 30 min and washed again in ice-cold PBS to stop the reaction.

RNA-sequencing (RNA-seq) and gene set enrichment analysis

The RNA-seq libraries were prepared using the TruSeq Stranded Total RNA Sample Preparation Kit (Illumina) according to the manufacturer's instructions and were sequenced on an Illumina NovaSeq platform to generate 100 nt single-end reads. For further details, see *Data S1*.

Transplantation analysis, cell migration and cell fusion assays

TA muscles of recipient mice were pre-injured with 50 μL of CTX 48 h prior to the cell transplantation. 2×10^6 GFP-labelled myogenic cells (Cellomics Technology) in 10 μl growth medium were intramuscularly transplanted into the injury sites. The injection was performed with a Hamilton syringe (VWR72310-316). The needle was injected into the proximal TA muscle.

Primary cells were plated, and the cell density reached 70%. CytoSMART2 records cell migration and fusion data during the 48-h differentiation of primary myoblasts. Measure the movement of primary cells (30 cells for each genotype) by ImageJ.

Statistical analysis

Data are expressed as mean ± SEM. Unpaired Student's *t*-test and one-way analysis of variance (ANOVA) with Bonferroni correction were performed using SPSS software 20.0. *P* value < 0.05 indicates a statistically significant difference. Data were expressed as the relative values to control group.

Results

Analysis of mThy1-hSNCA mice muscle phenotype

The gross morphology and weight of muscle in 6m-mThy1-hSNCA mice are indistinguishable from that of their wild-type littermates, but the fast-twitch muscle, including TA and gastrocnemius (GAS), were significantly decreased in 20m-mThy1-hSNCA (*Figure S1A*). Histological analysis showed that the fibre diameter in 20m-mThy1-hSNCA TA muscle were decreased significantly, and a large number of myofibres with irregular shape and nuclear migration to the centre in muscle, and the phenomenon of muscle lysis was also observed (*Figure S1B,E*). We further observed increased fibrosis and decreased glycogen deposition in mThy1-hSNCA muscle by Sirius red staining and periodic acid Schiff (PAS) staining, respectively (*Figures S1F,G and S2A*). In addition, the expression level of myosin-heavy-chain (MyHC)-I increased in 6m-mThy1-hSNCA mice, whereas MyHC-II decreased (*Figure S2B–E*).

α-Syn is enriched in motor neuron axons and NMJs in muscle

Immunohistochemical results showed that overexpressed α-Syn protein formed aggregates in mThy1-hSNCA muscle, and the expression of pS129-α-Syn also increased (*Figure 1A*). In addition, compared with 6m-wild-type muscle, α-Syn aggregates were increased in 20m-wild-type muscle (*Figure 1A*). Surprisingly, neither hα-Syn nor α-Syn protein was expressed in the primary myoblasts; on the contrary, it was highly expressed in non-myogenic cells composed of fibroblasts, endothelial cells and neuronal cells (*Figure 1B*). Therefore, we co-stained with α-Syn/hα-Syn and NF200 (neurofilament protein) antibody. Consistent with the western blot results, α-Syn/hα-Syn is expressed in muscle neurons, and the myofibre itself does not express (*Figure 1C,D*). In addition, the number of NF200^{+} in 6m-mThy1-hSNCA muscle were indistinguishable from wild type, but decreased at 20m; interestingly, the axons of 6m-mThy1-hSNCA mice appeared vacuolated (*Figure 1E,F*), indicating that α-Syn overexpression did not affect the number of neurons that regulate skeletal muscle function in the early stage, but cause neuronal degeneration.

α-Syn aggregation leads to NMJ degeneration and causes intramuscular oxidative stress

TEM showed that there were many swollen mitochondria and damaged cristae in the intramuscular axons and NMJs of mThy1-hSNCA mice, and the autophagic vesicles were also increased significantly; in addition, the number of vesicles in the NMJ decreased by about 2.7-fold (*Figure 2A,B*). The results of the AAV9-hSyn-ACh2.0 probe tracer[S7] showed that the ACh secreted in mThy1-hSNCA muscle was decreased, and the proportion of acetylcholine receptors (AChR) fragmentation were increased (*Figure 2C,D*). Although we observed that the NMJs in mThy1-hSNCA mice showed the nuclei tended to decrease, however, almost no neurons disappeared completely (*Figure S3A–C*). Consistent with *Figure 1E,F*, the Caspase3^{+} neurons in mThy1-hSNCA muscle have no significant changes compared with the wild type (*Figure S3A,D*).

We performed genome-wide RNA-Seq analysis on 6m-WT and 6m-mThy1-hSNCA TA muscle. As expected, gene sets associated with NMJ activity, neurotransmission and regulation of ROS metabolic process were highly enriched in mThy1-hSNCA (*Figures 2E and S3E*). Further analysis revealed that these genes that regulate ROS metabolism were involved in the glutathione pathway (*Figure S3F*). The expression of most genes (Hsp90aa1, Nos3, Cyp1b1) regulating ROS metabolism was reduced in mThy1-hSNCA muscle, except for GSTT2 and GSTM1 (*Figure S3G*). GSTT2 and GSTM1 belong to a superfamily of glutathione S-transferases that metabolizes a broad range of ROS. The higher activity of GSTT2 and GSTM1 in mThy1-hSNCA muscle might be due to cope with increased oxidative stress during α-Syn aggregation. Subsequently, we found the expression level of NMJ-related genes (Musk, Hadc4, Chrna9) were decreased in mThy1-hSNCA, whereas Agrn was up-regulated (*Figure S3G*). In addition, protective regulators of neurogenic skeletal muscle atrophy (Gadd45α) and factors regulating muscle cell migration and differentiation (Egr1, Cxcl1) were significantly down-regulated, which further supports that overexpression of α-Syn leads to NMJ degeneration and causes intramuscular oxidative stress, ultimately resulting in muscle atrophy.

α-Syn aggregation causes intramuscular oxidative stress that induces myofibre apoptosis

TEM showed that the sarcomere in 6m-mThy1-hSNCA muscle was significantly shorter than wild type and the width of I band, H zone and Z line were also decreased (*Figure 3A,B*). The mitochondria cristae were broken and contained a lot of fragments in mThy1-hSNCA muscle. The quantitative analysis results showed that the protein oxidation was increased in mThy1-hSNCA (*Figure 3C*). The GSH/GSSG ratio and ATP level were reduced (*Figure 3D,E*). In addition, the protein level and enzyme activity of complex I were down-regulated (*Figures S4A and S6B*), and the 4E-BP protein was up-regulated in mThy1-hSNCA; conversely, the level of the ROS scavenger SOD1 was not changed (*Figure S4A*). ROS production from mitochondrial in mThy1-hSNCA muscle was elevated by 2.2-fold (*Figure 3F*). Next, we found that Caspase 3[+] nuclei were significantly increased in mThy1-hSNCA (*Figure S4C*).
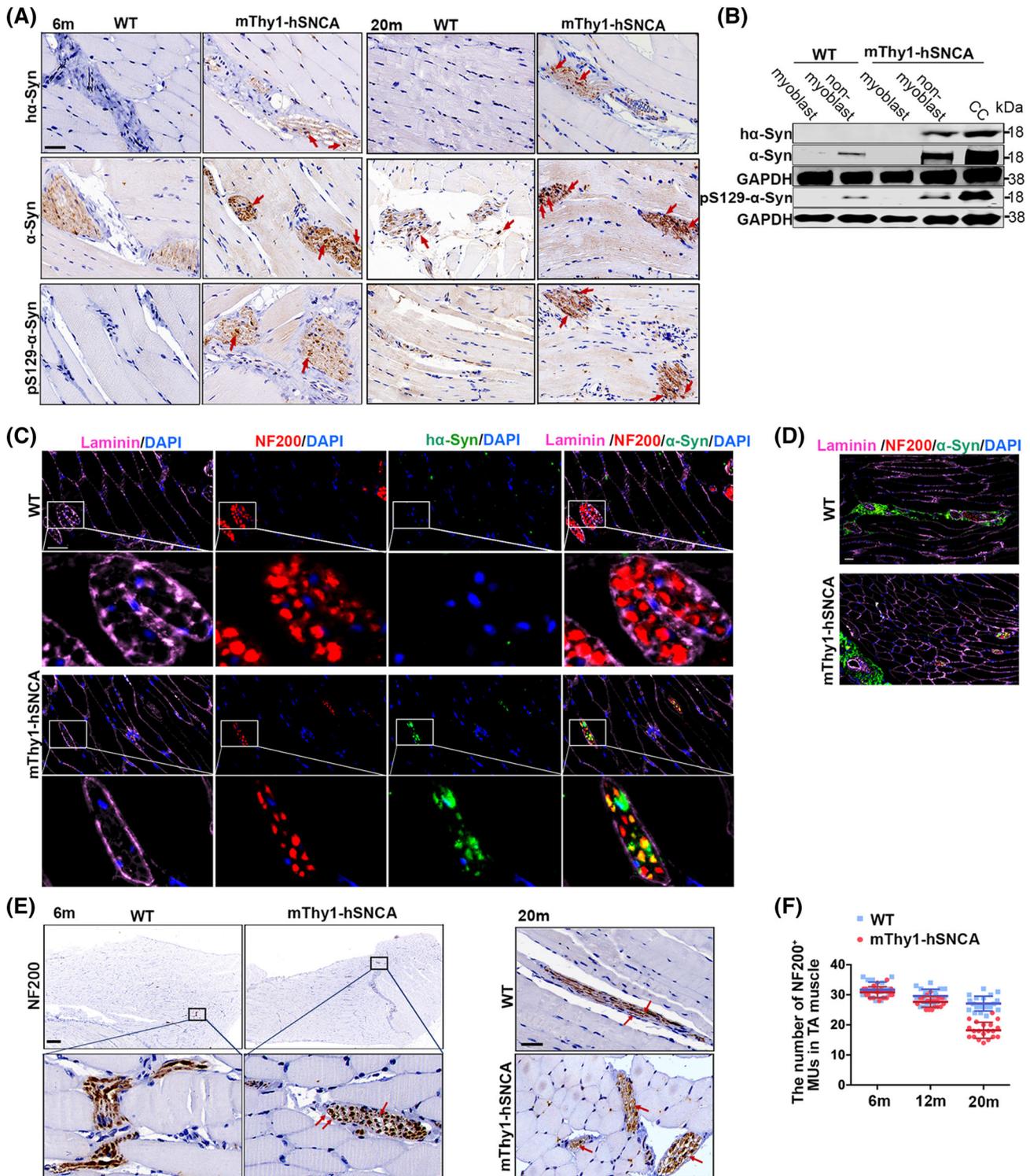
Figure 1 α-Syn is highly expressed in intramuscular motor neurons. (A) Immunohistochemistry of hα-Syn, α-Syn and pS129-α-Syn in 6-month-old and 20-month-old mice TA muscle, bar 20 μm, red arrow: α-Syn aggregation. (B) Western blot detecting hα-Syn, α-Syn and pS129-α-Syn protein expression in primary muscle cells; CC, cerebral cortex; non-myoblast: composed of fibroblasts, endothelial cells, neuronal cells and mesenchymal cells. (C) Immunofluorescence detection of co-localization of hα-Syn (green) and NF200 (red) in 6-month-old mice TA muscle, bar 60 μm. (D) Immunofluorescence detection of co-localization of α-Syn (green) and NF200 (red) in 6-month-old mice TA muscle, bar 60 μm. (E) Immunohistochemistry of NF200 in 6-month-old and 20-month-old mice TA muscle, 6m: bar 500 μm, 20m: Bar 20 μm, red arrow: vacuolated axon. (F) Quantification of the motor neurons that positively express NF200 at different ages. Five to six animals of each group were quantified. Data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$.
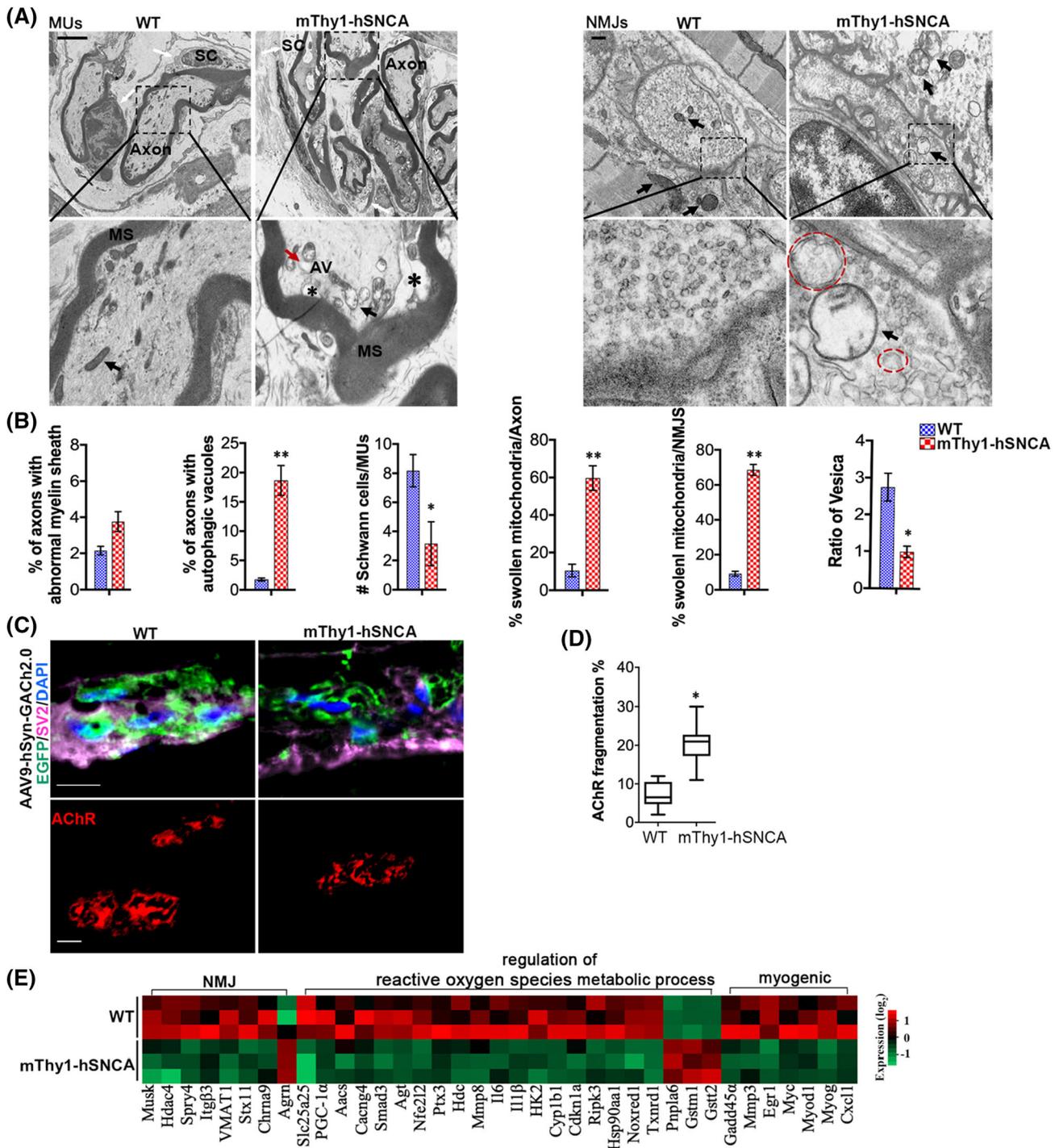
Figure 2 Neuromuscular junctions (NMJs) are degenerated in mThy1-hSNCA mice muscle. (A) Abnormal mitochondrial and autophagic vacuoles (AVs) in 6-month-old mThy1-hSNCA mice axon and NMJs, bar 2 μm, red arrow: AVs, black arrow: mitochondrial, white arrow: Schwann cells (SCs), red dotted circle: cisternae, black asterisks indicate ballooning of the myelin sheath (MS). (B) Percentage of myelinated axons present in the motor unit neuron (MUs) showing disintegration of their myelin sheaths and AVs in their axonal component; the number of Schwann cells in MUs; percentage of swollen mitochondrial in axon; percentage of swollen mitochondrial in NMJs; the ratio of Vesica in NMJs. (C) Representative images of ACh (EGFP, green; nerve terminal markers, SV2, pink) after injection of AAV9-hSyn-GACh2.0; images show the AChR (red) in 6-month-old wild-type and mThy1-hSNCA mice TA muscle, bar 50 μm. (D) Percentage of AChR fragmentation in TA muscle. (E) Heat map of FPKM values from wild-type and mThy1-hSNCA mice TA muscle. Red indicates increased expression, and green indicates decreased expression. Three to five animals were measured for each group. Data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$.
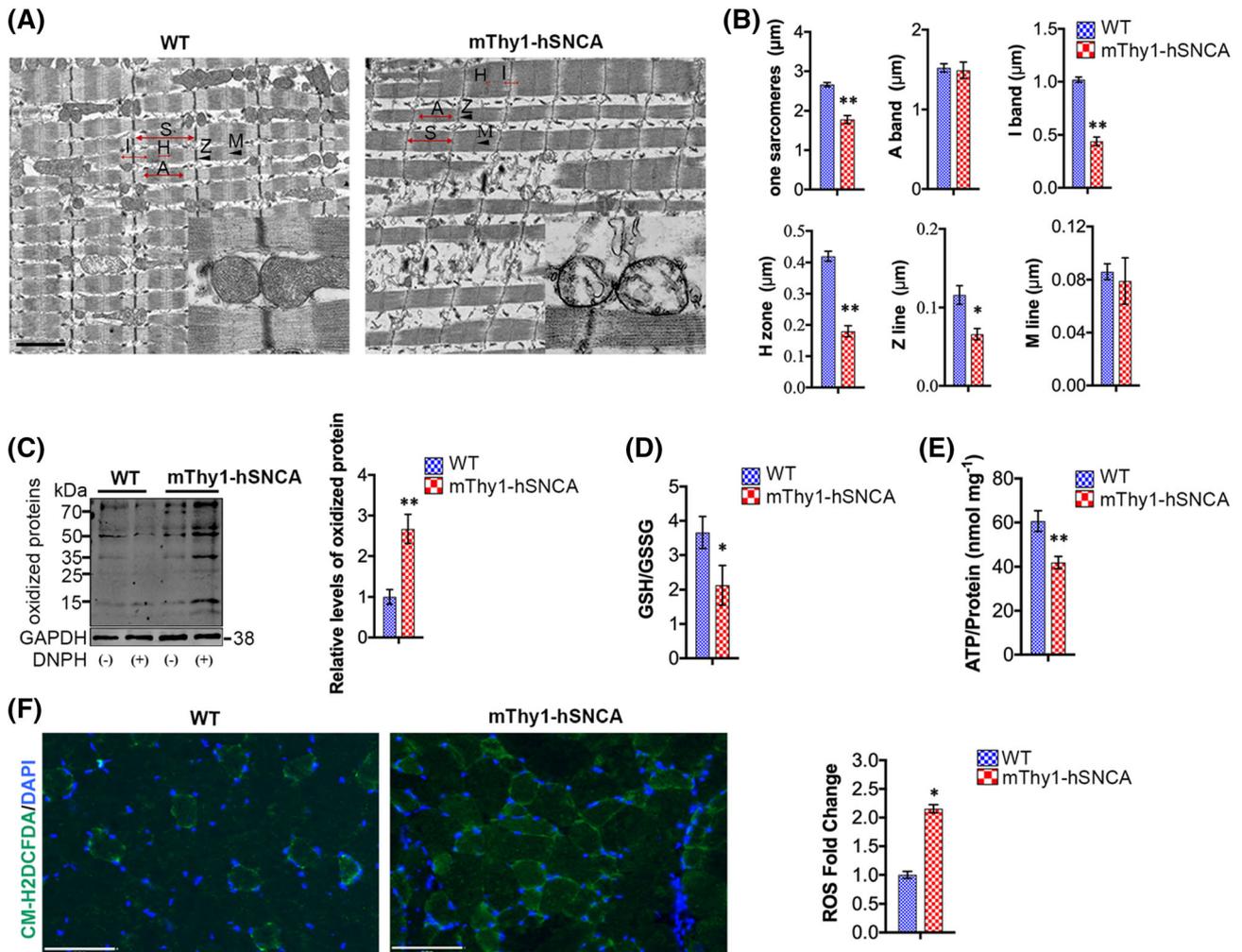
Figure 3 Overexpression of α-Syn impairs mitochondrial function. (A) TEM images of the 6-month-old wild-type and mThy1-hSNCA mice TA muscle, bar 2 μm, double arrow: sarcomere (S), A band, I band, H zone; black arrow: Z line and M line. (B) Quantification of the sarcomere, A band, I band, H zone, Z line and M line. (C) OxyBlot assay to detect protein oxidation in 6-month-old wild-type and mThy1-hSNCA mice TA muscle. (D) GSH/GSSG measurement in 6-month-old wild-type and mThy1-hSNCA mice TA muscle. (E) ATP measurement in 6-month-old wild-type and mThy1-hSNCA mice TA muscle. (F) Detection of intracellular ROS in 6-month-old wild-type and mThy1-hSNCA mice TA muscle, bar 60 μm. Three to five animals were measured for each group. Data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$.

These results suggest that overexpression of α-Syn causes chronic oxidative stress, suppressing ROS scavenging activity, and damage mitochondrial cristae structure in mThy1-hSNCA muscle, which is important for myocyte survival.

α-Syn aggregation causes intramuscular oxidative stress that delays muscle regeneration

The oxidative stress state of mitochondria in mThy1-hSNCA muscle prompted us to identify whether α-Syn regulates muscle regeneration. We used the well-characterized cardiotoxin (CTX)-induced injury model. Histological analysis at various time points after CTX injection revealed delayed regeneration of muscle in mThy1-hSNCA mice. The distribution of fibres

with concentrated nuclei inclines to smaller myofibres (*Figure 4A–C*). The percentage of regenerated fibres containing two or more central nuclei also decreased significantly at 7d (*Figure 4D*). The newly generated myofibre of mThy1-hSNCA mice was smaller, and fibrosis significantly increased when compared with wild type at both 14d and 21d post-CTX injection (*Figure 4A*).

During muscle regeneration, we observed that the newly formed eMHC+ myofibre size was significantly smaller (*Figure 4E*) and the expression of eMHC and myogenic transcription factors (Myod and Myogenin) (*Figure 4F*) was decreased in mThy1-hSNCA muscle 3d post injury, suggesting delayed or diminished myogenic cell expansion. Interestingly, the expression of Myf5, an early myogenic transcription factor that mark proliferating myoblasts, was not altered in
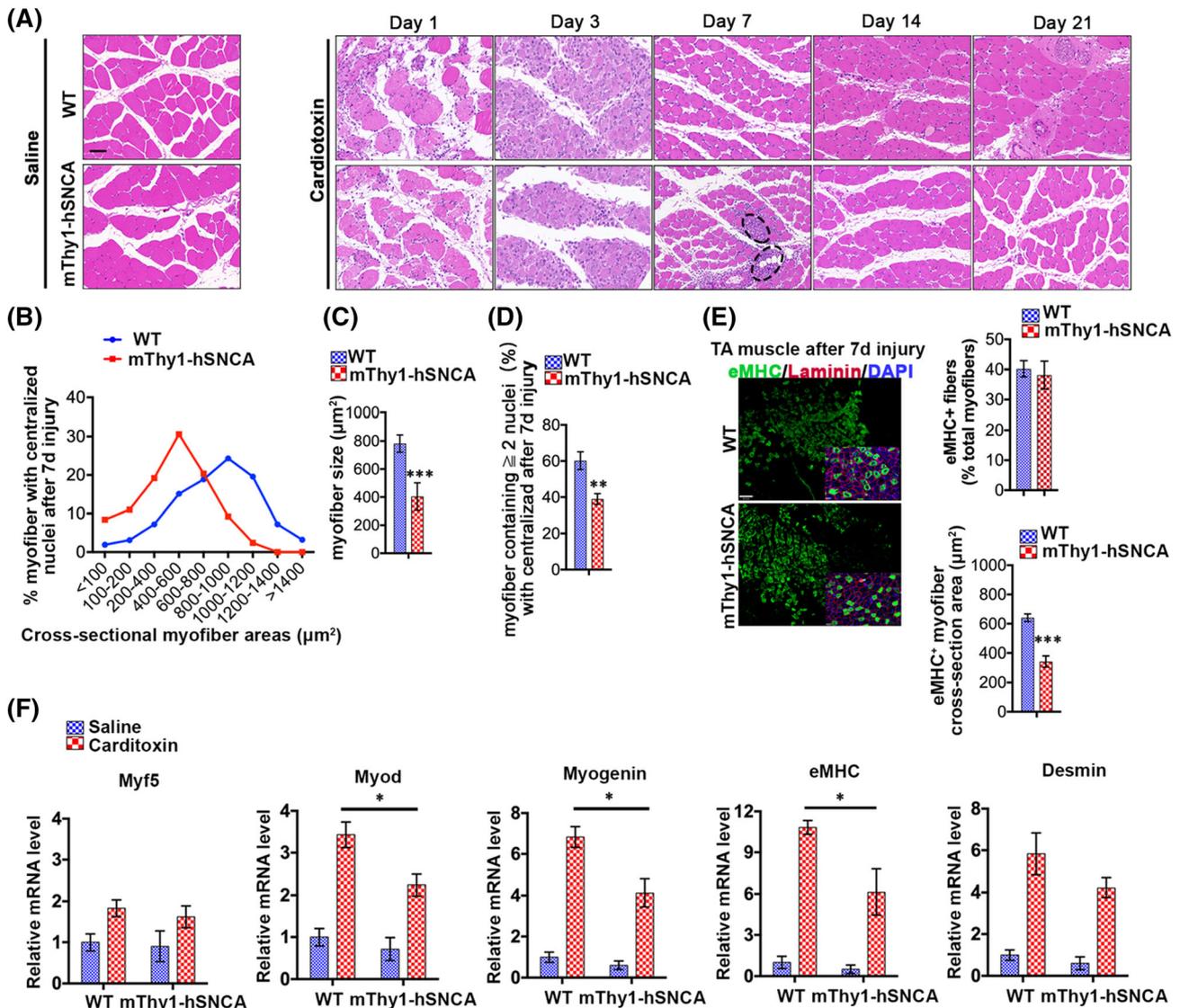
Figure 4 Delayed muscle regeneration in mThy1-hSNCA mice after cardiotoxin (CTX) injection. (A) H&E staining of transverse sections of the 6-month-old wild-type and mThy1-hSNCA TA muscles at days 1, 3, 7, 14 and 21 after CTX injury, bar 60 μm, black dotted circle: aggregated inflammatory cells. (B and C) regenerating myofibre size (percentage) distributions and area average of WT and mThy1-hSNCA TA muscle 7d after CTX-mediated injury were measured by using ImageJ software. Only myofibres that contained centrally located nuclei were counted. (D) Quantification of the ratio of regenerating myofibres containing two or more centralized nuclei per field at day 7 post injury. (E) Representative overlaid photomicrographs of TA muscle sections of WT and mThy1-hSNCA mice 7d post injury after immunostaining for eMHC (green) and laminin (red), nuclei were labelled by DAPI, bar 160 μm. Quantitative analysis of total regenerating (eMHC$^+$) myofibre number (as % of total myofibres) and average area of eMHC-positive fibres in TA muscle 7d post injury. (f) Real-time PCR shows the expression of myogenic markers in TA muscle 3d after CTX injury. Four animals of each group were quantified. Data are presented as mean ± SEM. $*P < 0.05$, $**P < 0.01$.

mThy1-hSNCA muscle (*Figure 4F*). Together, our results suggest that overexpression of α-Syn in mice significantly impairs the regeneration of adult muscle.

α-Syn aggregation alters the MuSC metabolic properties

The obvious defect of muscle regeneration in mThy1-hSNCA mice prompted us to investigate whether the function of MuSCs were affected. A similar number of Pax7-positive MuSCs were observed in 6m-wild-type and 6m-mThy1-hSNCA muscle (*Figure 5A*). To confirm the frequency of MuSCs production in mThy1-hSNCA muscle, we analysed FACS purified MuSCs from normal and after 1d injured muscle using well-established surface markers (CD45$^-$/CD31$^-$/Sca-1$^-$/CXCR4$^+$) (*Figure 5B*). The ultrastructure showed that the number of swollen mitochondria increased in MuSCs from mThy1-hSNCA mice (*Figure 5C*). Compared with wild type, the proportion depolarization of mitochondrial
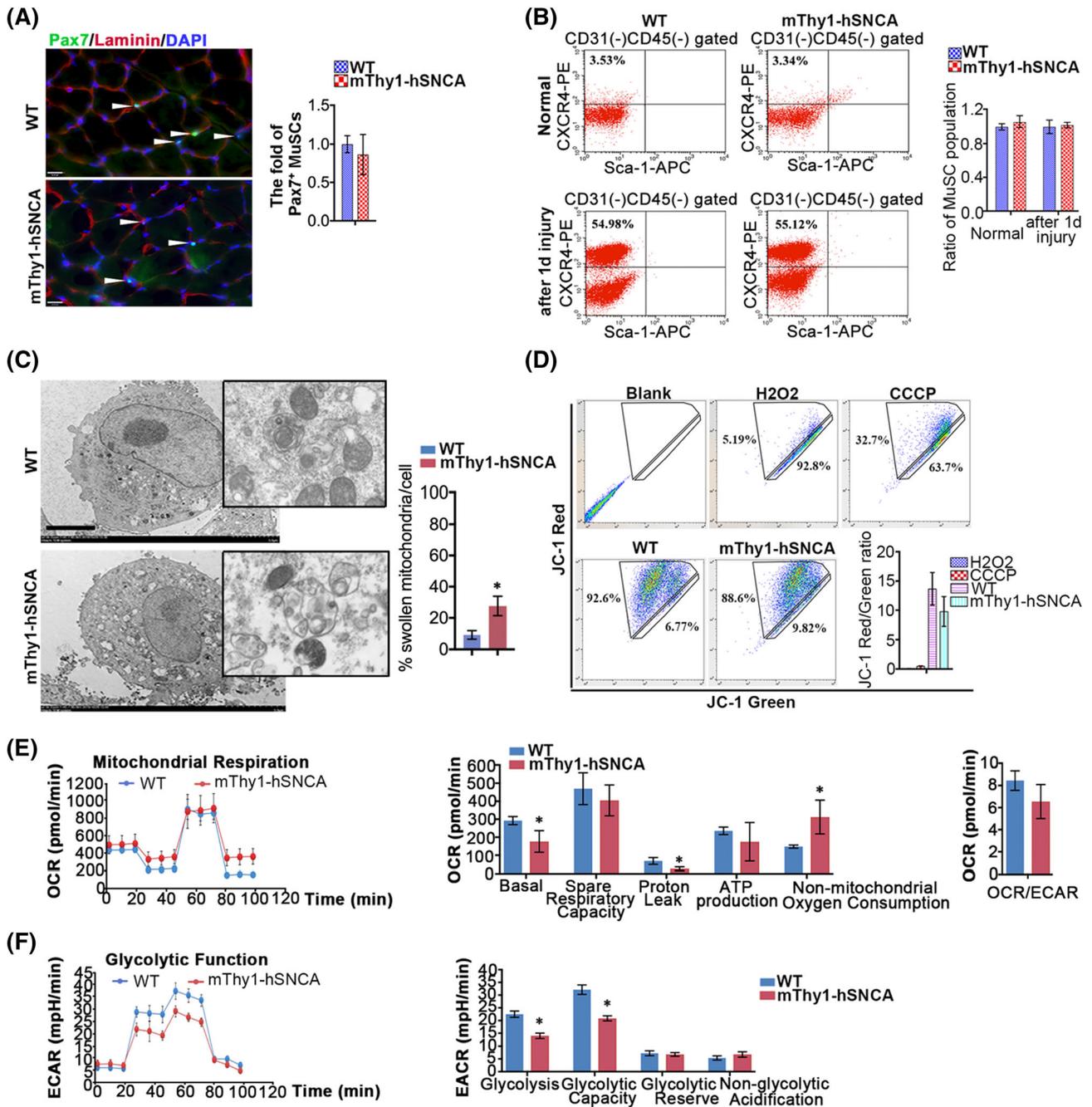
Figure 5 The regeneration pool and proliferation ability of muscle satellite cells (MuSCs) in mThy1-hSNCA mice remained unchanged, but their metabolic properties were altered. (A) Immunofluorescence staining to detect MuSCs in 6-month-old wild-type and mThy1-hSNCA mice TA muscle, and quantification of the Pax7+ MuSCs in TA cross-sectional; Pax7 (green) laminin (red), white arrow head: Pax7+ MuSCs, bar 32 μm. (B) Flow cytometry detection the number of MuSCs in 6-month-old wild-type and mThy1-hSNCA mice normal TA muscle and muscle 1d after injury, CD31−CD45−Sca-1-−CXCR4+. (C) TEM images of the MuSCs and quantification of swollen mitochondria in MuSCs, bar 1 μm. (D) JC-1 mitochondrial membrane potential staining of MuSCs. (E) Mitochondrial respiration capacity of MuSCs. (F) Glycolytic capacity of MuSCs. Four to five animals of each group were quantified. Data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$.

transmembrane potential in mThy1-hSNCA mice detected by JC-1 tends to increased, but there was no significant difference between the two groups (*Figure 5D*). Finally, the MuSCs of mitochondrial OCR from wild type and mThy1-hSNCA was

detected. As shown in *Figure 5E*, the basal respiratory capacity and ATP production of cells from mThy1-hSNCA mice were decreased; on the contrary, non-mitochondrial oxygen consumption was increased significantly. We next determined

the OCR/ECAR ratio to assess the relative contribution of glycolysis and mitochondrial respiration to energy generation. The OCR/ECAR ratio showed preference for OXPHOS in the wild type compared with mThy1-hSNCA MuSCs, indicating that the mitochondria of the wild-type MuSCs have a greater potential for substrate oxidation and ATP turnover (*Figure 5E*). In addition, as shown in *Figure 5F*, the glycolysis capacity of MuSCs from mThy1-hSNCA reduced 1.5-fold when oligomycin was added. These findings suggest that overexpression α-Syn causes oxidative stress in muscle, which alters the metabolic profile of MuSCs.

α-Syn aggregation causes intramuscular oxidative stress that inhibits myogenic differentiation during muscle regeneration

The above results prompted us to examine the myogenic fate of MuSCs in mThy1-hSNCA mice during muscle regeneration. There were usually a large number of proliferating and differentiating cells at 3d post injury. Interestingly, the number of Pax7$^+$/MyoD$^+$ and Pax7$^-$/MyoD$^+$ cells (*Figure 6A,B*) and the protein levels of MyoD and Myogenin (*Figure 6C*) were significantly reduced in mThy1-hSNCA. The genetic ontology analysis of biological processes demonstrated that overexpression of α-Syn causes many genetic changes that encode myoblast migration and cell fusion. qPCR identified most of these chemokines, and their receptors were significantly reduced (*Figure S5A,B*).

The differentiation status of the above cells within 48 h was tracked by video microscope. Compared with the wild type, the migration distance of primary myoblasts isolated from mThy1-hSNCA mice was significantly shorter, and the speed slowed down; in addition, the cells gathered in clusters delayed fusion and differentiation (*Figure 6D,E* and *Movies S1-2*). In order to further identify the cell migration ability during muscle regeneration in mThy1-hSNCA mice, we transplanted C2C12-GFP cells into the TA muscles at the same position post 2d injury (*Figure 6F*). The results showed that GFP$^+$ cells presented a diffuse distribution in wild-type mice, whereas GFP$^+$ cells gathered locally in mThy1-hSNCA muscle at the proximal end and a few GFP$^+$ cells at the distal end. In summary, these results indicate that overexpression of α-Syn inhibits the differentiation phase of muscle regeneration process.

Mito-TEMPO can relieve oxidative stress in mThy1-hSNCA mice

Mito-TEMPO (MT) is a mitochondrial-targeted superoxide dismutase, which removes superoxide and improves mitochondrial ROS in ageing muscles.[S8] In order to evaluate the effect of MT on improving the oxidative stress in

mThy1-hSNCA muscle, 20 mg/kg of MT was injected twice a week into mThy1-hSNCA mice TA for 21d, and the levels of ROS and oxidized protein produced by mitochondria in muscle were significantly reduced (*Figure 7A,B*), the ratio of GSH/GSSG and the production of ATP in muscle were up-regulated (*Figure 7C,D*). Correspondingly, the complex I/V protein levels and enzyme activities in mThy1-hSNCA-MT mice were significantly increased (*Figure S6A–C*), but the muscle mass did not increase (*Figure S6D*). We further observed that the sarcomere length of mThy1-hSNCA-MT mice did not alter, but the width of I band and Z line increased; in addition, the swollen mitochondria in the motor units (MUs) and NMJs had a tendency to decrease, and the autophagic vesicles in axons were also significantly reduced (*Figure 7G,H*), the number of fragmented AChR decreased, and the level of ACh secreted slightly improved (*Figure S6E,F*). Quantitative analysis of proteins that regulate reactive oxygen metabolism pathways showed that the levels of PGC-1a, SOD1 and Cyp1b1 were all up-regulated after MT treatment; on the contrary, when the total AMPK protein did not alter, the level of P^{Thr172}-AMPK, which is abnormally activated by ROS, was reduced and the expression of Caspase3 was also reduced (*Figure S6G, H*). The ratio of depolarization of muscle cell transmembrane potential detected by JC-1 had a trend of recovery (*Figure S6I,J*). These results indicate that MT treatment can restore the intramuscular oxidative stress state of mThy1-hSNCA mice.

MT can repair skeletal muscle aplastic disorder in mThy1-hSNCA mice

To determine whether MT can improve muscle regeneration in mThy1-hSNCA mice. We then injected CTX into mThy1-hSNCA mice treated with MT. Compared with the mThy1-hSNCA-Saline group, the MT-mThy1-hSNCA group had a significant increase in new myofibres with a central nucleus >2 after 7d of injury, and the new myofibre size increased significantly after 21d of injury (*Figure 8A–D*). We further quantitatively analysed the expression of myogenic factors post 7d injury; the results showed that Pax7, Myogenin and eMHC did not change significantly in the mThy1-hSNCA-MT group compared with the mThy1-hSNCA-Saline group, whereas the chemokines CCL6 and CCL9 in the latter were up-regulated (*Figure 8E,F*). Next, we transplanted C2C12-GFP cells into mThy1-hSNCA-MT group TA muscle according to the procedure of *Figure 6F*. It was observed that GFP$^+$ cells migrated to the surrounding area post 5d transplantation compared with the mThy1-hSNCA-Saline group (*Figure 8G*). The above results suggest that MT can improve the migration ability of MuSCs during muscle regeneration in mThy1-hSNCA mice.
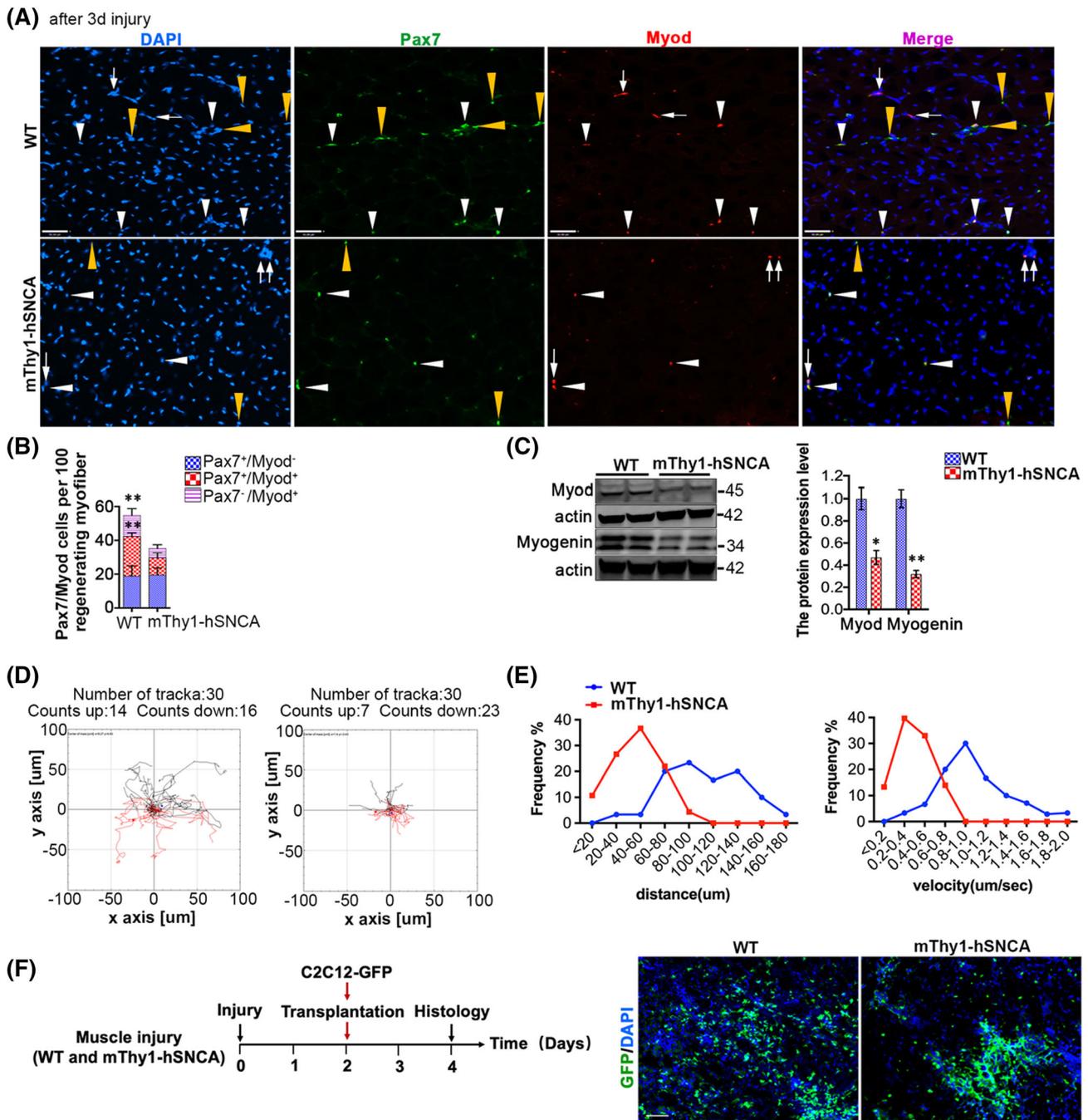
Figure 6 Inhibition of myogenic differentiation during muscle regeneration in mThy1-hSNCA mice. (A) Immunofluorescence staining to detect myogenicity of MuSCs in 6-month-old wild-type and mThy1-hSNCA mice TA muscle after 3d post injury, Pax7 (green)/Myod (red)/DAPI (blue), yellow arrow head, Pax7$^+$/Myod$^-$ cells, white arrow head, Pax7$^+$/Myod$^+$ cells, white tail arrow, Pax7$^-$/Myod$^+$ cells, bar 60 μm. (B) Measure the number of Pax7$^+$Myod$^-$/Pax7$^+$Myod$^+$/Pax7$^-$Myod$^+$ cells. (C) Western blot detecting myogenic factors (Myod and Myogenin) protein expression levels in TA muscle after 3d injury. (D and E) Quantification of cell migration distance. (F) Detection of GFP$^+$ cell migration distance in 6-month-old wild-type and mThy1-hSNCA mice TA muscle after transplantation 2 days, bar 100 μm. Four animals of each group were quantified. Data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$.

Discussion

Our results reveal for the first time that the pathogenic α-Syn protein plays an important role in regulating intra-

muscular mitochondrial oxidative stress, leading to muscle atrophy. Mitochondrial dysfunction in the skeletal muscle of mice overexpressing α-Syn leads to myofibre apoptosis and muscle regeneration after CTX injury is significantly
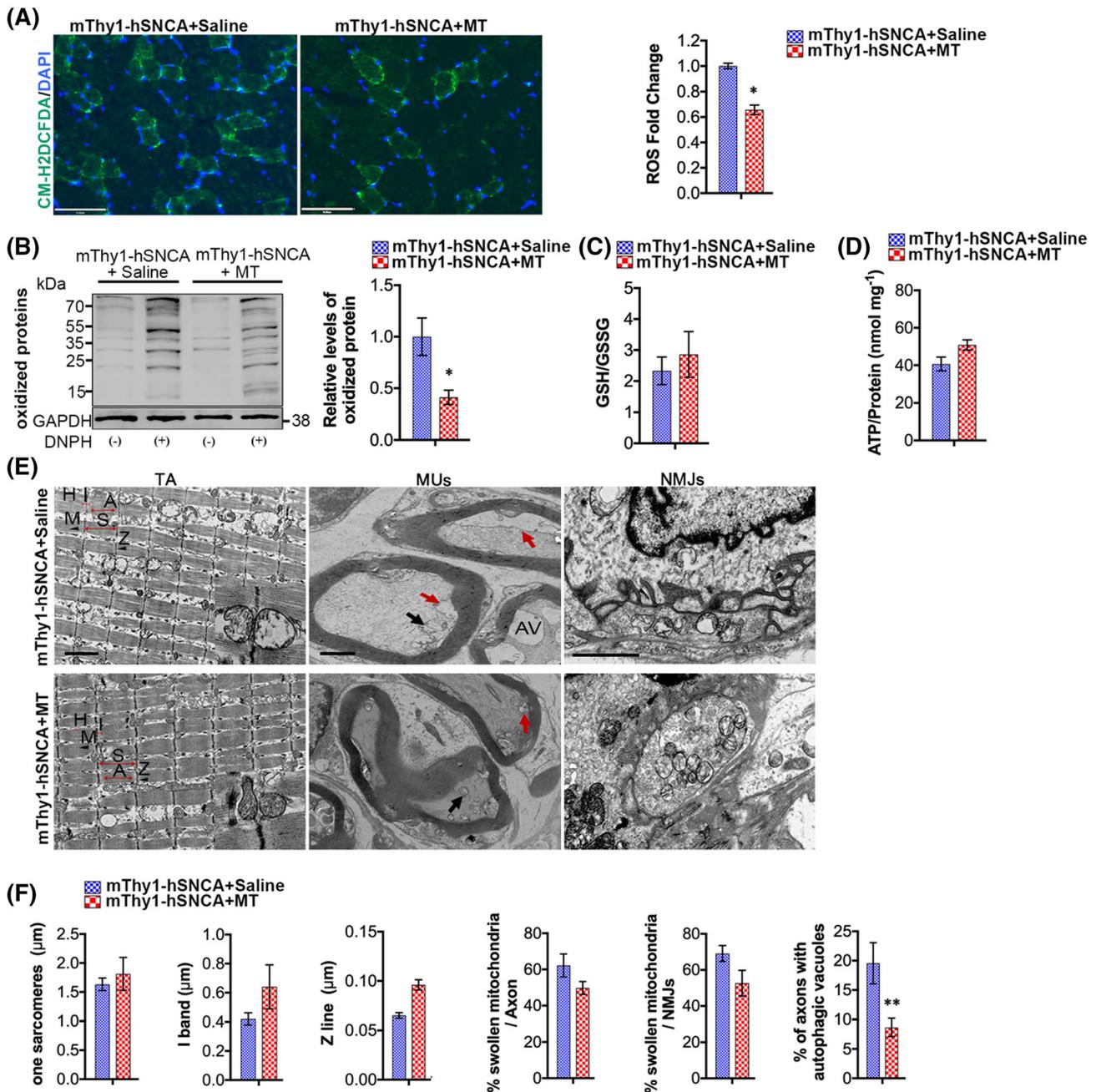
Figure 7 Mito-TEMPO (MT) relieves intramuscular oxidative stress in mThy1-hSNCA mice. (A) Detection of intracellular ROS in 6-month-old mThy1-hSNCA-Saline and mThy1-hSNCA-MT mice TA muscle, bar 60 μm. (B) OxyBlot assay to detect 6-month-old mThy1-hSNCA mice TA muscle protein oxidation after 21d MT treatment. (C) GSH/GSSG measurement in 6-month-old mThy1-hSNCA mice TA muscle for 21d MT treatment. (D) ATP measurement in 6-month-old mThy1-hSNCA mice TA muscle for 21d MT treatment. (E) TEM images of mitochondrial in axon and NMJs for 21d MT treatment, black arrow: mitochondrial, red arrow: autophagic vacuoles (AVs), bar 2 μm, double arrow: Sarcomere (S), A band, I band, H zone; black arrow: Z line and M line. (F) Quantification of the sarcomere, I band and Z line by 21d MT treatment; percentage of the swollen mitochondria and AVs in axon and NMJs. Three to five animals were measured for each group. Data are presented as mean ± SEM. *: WT and mThy1-hSNCA, $: mThy1-hSNCA and mThy1-hSNCA-MT; *$P < 0.05$, **$P < 0.01$.

delayed. This is largely due to the oxidative stress of intramuscular mitochondria that has transformed the metabolic properties of MuSCs. In terms of mechanism, we found that α-Syn protein accumulates in NMJs and motor neuron axons in skeletal muscle can inhibit the ACh release of NMJ presynaptic vesicle and increase intramuscular oxidative stress including increased intracellular ROS and decreased ATP synthesis. These data can provide new insights for muscle dysfunction and atrophy in PD and synucleopathies.
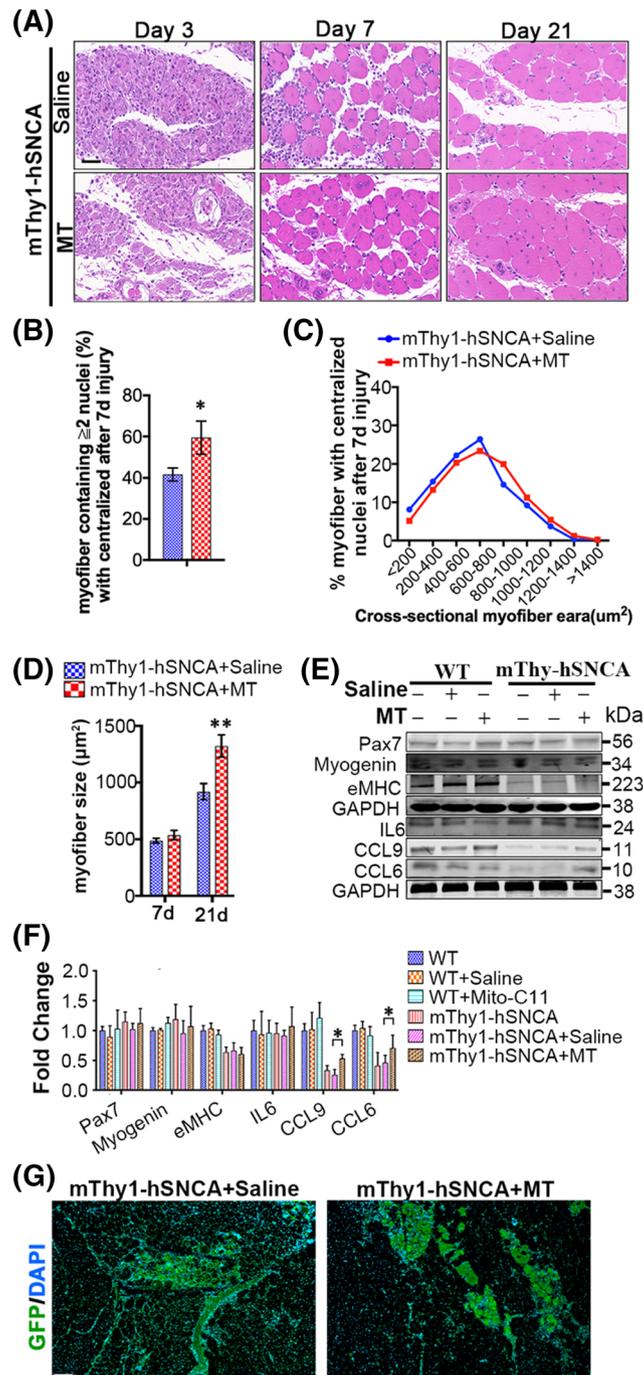
Figure 8 The muscle regeneration of mThy1-hSNCA mice can be improved by MT treatment. (A) H&E staining of transverse sections of 6-month-old mThy1-hSNCA-saline and mThy1-hSNCA-MT TA muscles at day 3, 7 and 21 after CTX injury, bar 60 μm. (B) The ratio of regenerating myofibres containing two or more centralized nuclei per field at 7d post injury. (C and D) Regenerating myofibre size (percentage) distributions and area average of mThy1-hSNCA-Saline and mThy1-hSNCA-MT TA muscle post 7d injury. (E and F) Western blot detecting the expression level of myogenic factors (Pax7, myogenin, eMHC) and chemokines (CCL6, CCL9). (G) Detection of GFP$^+$ cell migration distance in 6-month-old mThy1-hSNCA-saline and mThy1-hSNCA-MT mice TA muscle after transplantation 5 days, bar 160 μm. Four animals of each group were quantified. Data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$.

PD or synucleopathy is a common age-related syndrome with motor deficits. Researchers have established various animal models with the key pathogenic α-Syn protein. These animal models mimic the pathological phenotypes of PD, such as the aggregation and dissemination of α-Syn protein. The mouse models overexpressing α-Syn in a variety of different

promoters all showed muscle loss and movement disorder. For example, mutations in the A30P and A53T sites under the TH promoter could cause extensive peripheral neuropathy and muscle diseases in mice,[20] whereas mutation of the A30P site under the Nguyen virus promoter could cause dystonia and tremor in mice or hamsters.[21] Compared with wild type, our results found that 6m-mThy1-hSNCA mice had changes in skeletal muscle metabolism, increased myofibre apoptosis and delayed regeneration when there were no obvious motor and behaviour changes. Muscle strength in mThy1-hSNCA mice declined at 8m, α-Syn protein in dopamine neurons aggregated at 9m,[22,S9] and the movement disorders phenotype did not appear until 12m (data not shown). Notably, Martin et al[23] showed motor neurons were depleted (75%) in A53T mice (mutant human A53T a-Syn driven by PrP promoter), accompanied by skeletal muscle denervation and extensive muscle atrophy. They describe a reduction in slow twitch in A53T mice muscle, but they did not quantify the expression of MyHCs (MyHC-I and MyHC-IIa/b/x), nor whether severe muscle atrophy resulted in a dramatic reduction in all myofibres. In contrast, we observed a reduction in fast-twitch myofibre in mThy1-hSNCA mice. However, no significant reduction in motor neurons was observed in 20-m-mThy1-hSNCA mice, which is consistent with the findings of Mu et al[5] on the identification of skeletal muscle fibre types in Parkinson's patients. We speculate the reasons for the reduction of fast-twitch myofibre in mThy1-hSNCA mice: (i) Pathogenic aggregates formed by α-Syn are associated with increased susceptibility of NMJs on fast-twitch myofibres, such as increased endplate debris and decreased presynaptic and postsynaptic co-localization.[24] (ii) The degeneration of NMJs caused changes in the firing frequency of neurons. Motor neuron impulse frequency determines myofibre MHC expression. As denervated myofibres can undergo reinnervation by both low (innervate slow-twitch myofibres) and high (innervate fast-twitch myofibres) threshold neighbouring motor neurons,[25] the current results showed the characteristics of muscle dysfunction and atrophy earlier than the clinical motor phenotypes. The above results support the hypothesis that α-Syn protein overexpression accelerates muscle senescence. So far, because no specific preventive treatment of PD muscle atrophy has been established, sarcopenia preventive measures should be taken into consideration as early as possible.

Mitochondria play a key role in skeletal muscle metabolism and is very sensitive to physiological or pathological damages. Blin,[26] Cardell[10] and Wallace et al[S11] reported that PD patients had defects in mitochondrial complex I in fast muscles. Pathological observations found that intermuscular mitochondria of PD patients were decreased and most patients with PD have impaired OXPHOS of muscle mitochondria although there was no loss of mitochondrial DNA. The above data show that the pathological changes of PD muscular atrophy are mainly due to the significant increase in the production of peroxides in intramuscular mitochondria, which leads to oxidative stress. However, the mechanism of peroxides production and the reaction mechanism of muscles to these peroxides are still unclear. Consistent with the clinical observations, we found the mitochondria with swelling and cristae structure breakage between myofilaments in mThy1-hSNCA mice were increased significantly and the complex I activity and membrane potential were decreased. This caused an increase in ROS in mThy1-hSNCA muscle, which intensified oxidative stress and led to altered intramuscular metabolic niche. These results show that mThy1-hSNCA mice mimic the muscle pathological phenotype of PD patients and provide a feasible way for further research on the mechanism of muscle dysfunction in PD patients.

Interestingly, our results are similar to those reported by Putten[10] and Norihito[S12] that the α-Syn protein in mThy1-hSNCA mice is not only enriched in the brain, but also abundantly enriched in motor neuron axons and NMJs, but not in the myofibres themselves. We found that the number of NF200+ myelinated axons did not change until 20m; however, the number of presynaptic membrane vesicles of NMJs decreased significantly at 6m. Indeed, mThy1-hSNCA mice revealed a significant increase in phosphorylated α-Syn at Ser129 under basal/non-stimulated conditions, compared with the NMJs of WT. Chen and Feany[27,S13] found that phosphorylation of α-synuclein at Ser129 significantly enhanced α-synuclein toxicity in vivo in a *Drosophila* model of PD. Severe mitochondrial morphological changes were displayed in the NMJs of mThy1-hSNCA mice, revealing a correlation between mitochondria-driven ROS formation and increased p^{Ser129}-α-Syn. Consistently with our data, it was previously shown that α-Syn aggregation caused a multitude of mitochondrial defects, including decreased mitochondrial membrane potential and energy production, increased ROS, disruption of mitochondrial-ER Ca2 + homoeostasis, inhibited mitochondrial dynamics and induced mitochondrial pro-apoptotic protein cytochrome C release.[15,28,S5,S14] The above results indicate that overexpression of α-Syn protein causes chronic destruction of neuromuscular synapses and degeneration of NMJs and the degeneration will be aggravated with ageing. In this study, we used AAV9-hsyn-ACh2.0 probe[S7] to label endogenous ACh and found that ACh released by NMJ presynaptic membrane vesicles was reduced. It is known that ACh can regulate the FoxO3/PGC-1a pathway to activate SOD2 in mitochondria and up-regulate SOD1 in the cytoplasm, thereby inhibiting the production of ROS during oxidative stress.[29] The latest research results also found that the quantitative release ACh can activate the AChR, which could prevent muscle atrophy and promote innervation,[30] indicating that the neurotransmitter ACh released not only affects myofibre contraction but also plays an important role in regulating oxidative stress-induced muscle atrophy. Our results confirmed that α-Syn protein aggre-

gation inhibits the release of ACh from presynaptic vesicles of NMJs, causing oxidative stress in intramuscular mitochondria, which is a key factor leading to PD muscle atrophy. This is supported by Mu et al[5] found that α-Syn aggregates in the NMJs of the pharyngeal muscles of patients with sporadic PD and caused degeneration of axons and NMJs. However, it was observed that α-Syn accumulated mostly in the substantia nigra and other subcortical nuclei rather than the spinal cord and NMJs in the mThy1-hSNCA mice established by Rockenstein.[31] The differences in the aggregation and distribution of α-Syn in different PD animal models overexpressing hα-Syn[10] could be due to the different genetic backgrounds of these mice (C57 VS DBA) and different overall expression levels and transgene insertion site.

In our study, MT treatment could restore the oxidative stress status of mThy1-hSNCA mice and improve the ability of muscle regeneration. In addition, the NMJ receptor fragmentation and ACh secretion were also improved. MT is a mitochondrial-targeted antioxidant that can pass through the phospholipid bilayer and accumulate in mitochondria.[S8] Recent studies have found that MT can reverse ethanol-induced mitochondrial dysfunction (including oxygen consumption, ROS production and oxidative stress), increase tricarboxylic acid cycle (TCA), circulate intermediates and reverse the impaired protein synthesis and sarcopenia phenotype.[32] In our study, mitochondrial function was restored in skeletal muscle of mThy1-hSNCA mice after MT treatment, with increased Z line and I band width, suggesting that MT could improve muscle fibre contractility. MT treatment can improve the ability of muscle regeneration after injury; in addition, AChR fragmentation and ACh secretion are also improved, but sarcomere length and muscle mass are not increased, which may require extended treatment time. Consistent with reports from other studies,[32,S8,S15] MT partially restored the reduced myotube diameter and sarcopenic phenotype, suggesting that mitochondrial-targeted therapy may partially reduce mitochondrial ROS and allow maintenance of physiological function. Thus, the abnormal metabolism induced by α-Syn aggregation can be effectively restored. Beneficial response of MT targeting mitochondria lays a therapeutic basis for the use of mitochondrial ROS scavengers to reverse α-Syn aggregation-induced mitochondrial dysfunction and muscle loss in skeletal muscle.

Studies have reported that chronic destruction or degeneration of neuromuscular synapses, such as in muscular dystrophy[S16,S17] and biological ageing,[8,S18] will neutralize the interaction between MuSCs and motor neurons, resulting in obvious defects in muscle regeneration. These results emphasize the importance of NMJs and neural networks as essential for the myogenesis of MuSCs. We found that the regeneration pool of MuSCs in mThy1-hSNCA did not change; however, the migration and fusion of their myo-

blasts were inhibited. The results of RNA-Seq and quantitative analysis showed that the related chemokines that regulate cell migration and fusion were reduced in mThy1-hSNCA. Ultrastructural observations showed that the mitochondrial morphology altered in MuSCs from mThy1-hSNCA. It is well known that mitochondria reveal functional plasticity and capable of adapting to a variety of acute and chronic conditions, thus contributing to vulnerability or resilience of muscle cells.[33] Restriction of ATP production and reserve respiratory capacity reflecting overall mitochondrial dysfunction that are often associated with cell proliferation and differentiation have been demonstrated in sarcopenia[34] and neurodegenerative disease.[S19] Thus, the ability of the mitochondria to make ATP and to consume oxygen in response to energy demands serves as a reliable hallmark of its functional state, reflecting cell viability. Accumulating evidence indicates that mitochondria metabolism is not only a consequence of the MuSCs functional status but rather an active player that regulates MuSCs fate choices.[35,S20] Quiescent MuSCs display a low metabolic rate that mainly relies on fatty acid oxidation.[35,36] MuSC activation initially induces their glycolytic pathway in order to sustain cell proliferation,[36,S21] and then a switch to oxidative metabolism is required to allow further MuSC commitment to differentiation.[37,S21,S22] Consistent with this data, we found that the mitochondrial OCR of MuSCs in mThy1-hSNCA was reduced. Moreover, the process of MuSCs differentiation to form myotubes is highly dependent on OXPHOS metabolism. We also demonstrated that MuSCs derived from mThy1-hSNCA have reduced mitochondrial respiration rate, ATP production and glycolytic capacity. These data indicate that α-Syn-mediated oxidative stress changes the metabolic properties of MuSCs, which may be an important factor in inhibiting the differentiation of myoblasts and its self-renewal ability.

As we all know, α-Syn promotes the assembly of SNARE complex and interacts with synaptic vesicles to maintain vesicle operation and neurotransmitter release.[38] The latest research suggests that mitochondria participate in presynaptic energy metabolism to maintain synaptic signal transduction activities.[39] Mitochondrial OXPHOS provides the main energy source in nerve cells. Therefore, the transportation of axon mitochondria and the presynaptic positioning to maintain energy metabolism are essential for neurons to maintain high-intensity synaptic activity.[39] Studies have shown that α-Syn inhibited the activity of mitochondrial complex I in a dose-dependent manner and inhibits the production of mitochondrial oxygen and ATP.[19,S23] Our results confirm that α-Syn aggregation and dissemination induce oxidative stress and play an important role in the process of PD muscle atrophy. In the process of α-Syn aggregation, there are different intermediate conformations such as monomers, oligomers, protofibrils and inclusion bodies.[S24,S25] Although many previous studies have shown that the intermediate conformation

of oligomers and protofibrils has strong cytotoxicity, however, the transformation characteristics of α-Syn aggregation state and its mechanism of action in the process of PD muscle atrophy have yet to be determined. It is warranted to further explore how α-Syn protein aggregation targets or interacts with mitochondria to regulate the release of the presynaptic vesicle neurotransmitter ACh.

Conclusion

In this study, we found that the accumulation of α-Syn protein in NMJs caused intramuscular oxidative stress and muscle atrophy. These new findings indicate that PD is a systemic disease that not only involves dopaminergic neurons and glial cells in the brain but also involves peripheral immune cells and other organs, such as skeletal muscle. Muscle atrophy can also exacerbate the worsening and severity of PD. These results may provide experimental basis for effective intervention and treatment of clinical PD muscle atrophy in the future.

Acknowledgements

Conflict of interest

The authors declare no competing interests.

Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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