國立台灣大學醫學院微生物學研究所

碩士論文

Graduate Institute of Microbiology

College of Medicine

National Taiwan University

Master Thesis

探討肌肉表現之 NRIP 在運動神經元退化中的角色

The role of muscle-restricted expression of NRIP in

motor neuron degeneration

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中華民國 104 年 7 月

July, 2015

致謝

　　時光飛逝，碩士班生活即將告一個段落，感謝三年前陳小梨老師願意接受農學院背景出身的我進入 R710 實驗室學習，在老師的指導下以及每次的討論當中，都能感染到老師對於科學的那股執著與熱情，也謝謝老師的支持與鼓勵，讓我能夠在實驗之餘，把握時間充實自己，從您身上學習到很多，謝謝。感謝口試委員王培育老師、陳文彬老師、陳佑宗老師以及蔡力凱醫生在實驗上以及多次口試當中給了我諸多寶貴的建議及指教，讓這篇論文能夠更趨完整。也謝謝所上所有的老師課堂上的教導，讓我具備充足的專業知識。

　　謝謝 R710 大家庭的每個人，三年來我在這度過了很充實很愉快的時光。感謝思為學長在實驗技術上給我非常札實的訓練，也在我茫然摸索未來時給了我很多的建議以及實質上的幫助。感謝信雄學長在實驗上以及準備口試中的 cover，幫我節省很多自己摸索的時間。感謝楚嵐學長及立博學長在實驗室瑣碎事務上的幫忙，有你們在，減少了很多悲劇發生的機會。感謝孟萱學姊及思瑜學姊在實驗上還有生活上的經驗分享，跟你們聊天總是能增廣見聞。感謝東謙、怡柔、范萍在實驗上的幫忙，從你們身上我也學習到很多。最後感謝同班兩年的怡雯，總是跟你一起討論實驗討論生活討論未來，我有預感我們會是一輩子的好朋友。

　　謝謝所辦的助教們總是不辭辛勞叮嚀著我們重要的事項，謝謝在微生物所認識的所有同學、學長姐、學弟妹，因為有你們，生活不會只剩下實驗與 data，我會永遠記著曾經我們一起吃飯話家常，一起打球揮灑汗水的日子。

　　最後謝謝我最親愛的家人還有蔣昌華，你們就是我的避風港，不論是生活還是實驗遇到什麼開心不開心的事情，都能與你們分享，有你們作為後盾，我才可以盡情的做我想做的事情，謝謝你們。

中文摘要

核受體交互作用蛋白(Nuclear receptor interaction protein, NRIP)是本實驗室在 2005 年以雄性激素受體(Androgen receptor)作為餌，從 human HeLa cDNA library 中利用酵母菌雙雜合(yeast-two hybrid)選殖系統篩選而得。我們發現 NRIP 可作為轉錄調節上的共同活化子去調控 AR 下游基因的表現，另外，NRIP 也可在鈣離子存在情況下藉由與攜鈣素(calmodulin)之結合去調節肌肉的收縮運動。

從我們先前的研究中發現全身性 NRIP 基因剔除小鼠與正常小鼠相比，其肌肉強度較弱，且適應性運動行為表現也較差，此外，全身性 NRIP 基因剔除小鼠其腰部腹側脊髓的運動神經元也有缺失的現象。由於肌肉與運動神經元之間的關係十分緊密，能夠互相調控以維持正常的神經傳導及肌肉運動功能，另外，有文獻指出由肌肉所表現的一些因子能影響運動神經元的存活，因此在本篇論文中，我們想探討由肌肉所表現的 NRIP 對於運動神經元的存活所扮演的角色為何。

首先我們做出一隻肌肉 NRIP 基因剔除小鼠，其 NRIP 表現只會在肌肉組織中被剔除，肌肉以外的組織 NRIP 皆可正常表現。藉由免疫螢光染色發現肌肉 NRIP 基因剔除小鼠腰部腹側脊髓的運動神經元有缺失的現象，另外藉由全標本包埋染色法(whole-mounting staining)也發現肌肉 NRIP 基因剔除小鼠的神經肌肉接點(neuromuscular junction)有受到破壞的情形，代表肌肉表現的 NRIP 在維持運動神經元的存活以及神經肌肉接點的型態完整度中扮演著重要的角色。從行為實驗中，我們也進一步發現與正常小鼠相比，肌肉 NRIP 基因剔除小鼠表現出較差的運動能力。最後，我們想要探討肌肉是否會藉由 NRIP 去調控一些特殊因子的表現進而去調節神經肌肉接點的型態完整度以及運動神經元的存活，我們發現在肌肉 NRIP 基因剔除小鼠的比目魚肌中，成肌素(myogenin)的 mRNA 表現量會下降，而在感染 Ad-shNRIP 的 C2C12 細胞中，成肌素的 mRNA 表現也有下降的趨勢，這代表著

NRIP 的缺失會影響成肌素的表現量，因此我們認為在肌肉組織中 NRIP 能夠調控成肌素的表現量，並且，由肌肉所表現的成肌素在調控神經肌肉接點的型態完整度以及動神經元的存活可能扮演著重要的角色。

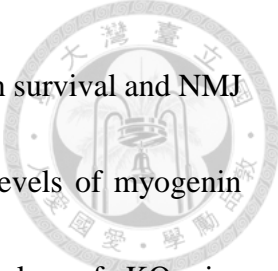關鍵字：核受體交互作用蛋白、運動神經元、神經肌肉接點、肌肉表現因子、成肌素。

Abstract

Nuclear receptor interaction protein (NRIP) is a novel nuclear receptor binding protein we identified by using AR as the bait to screen a human HeLa cDNA library in a yeast two-hybrid screening assay in 2005. NRIP functions as a transcriptional co-activator which mediates AR-driven gene expression and as a calcium-dependent calmodulin (CaM)-binding protein involved in muscle contraction.

From our previous study, we found conventional NRIP knockout mice have reduced muscle force and impaired adaptive exercise performance. Lumbar motor neuron loss in spinal cord was also observed in conventional NRIP knockout mice. Previous research suggested that muscle-expressing factors may regulate motor neuron (MN) survival and maintaining. Thus, we want to investigate the role of muscle-specific expression of NRIP in motor neuron survival.

First, we generated muscle-specific NRIP knockout mice, also named conditional KO (cKO) mice, as our experimental models. Results of immunofluorescence assay (IFA) and whole-mounting staining assay showed that cKO mice have less α-MNs and impaired neuromuscular junction (NMJ) in comparison to aged-matched WT mice, respectively. It implied that NRIP expressed by muscle plays an important role in maintaining motor neuron survival and NMJ integrity. From rotarod test, we also found cKO mice display deficient motor function compared with WT mice. Finally, we screened the muscle-

derived candidate factor(s) which is capable of affecting motor neuron survival and NMJ integrity by performing RT-qPCR reaction. We found that mRNA levels of myogenin which is a myogenic transcription factor are down-regulated both in soleus of cKO mice and in C2C12 cell infected with Ad-shNRIP. These results implied that NRIP may regulate the expression of myogenin and myogenin may be the muscle-derived factor that can affect motor neuron survival and NMJ integrity.
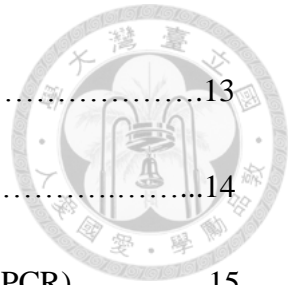
Key words: NRIP, Motor neuron survival, Neuromuscular junction, Muscle-derived factors, myogenin,

Table of Contents
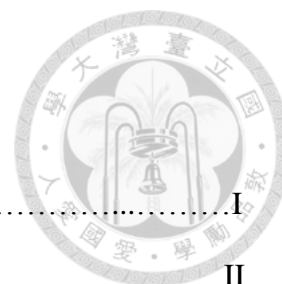
Chapter 1 INTRODUCTION

1.1 The characteristics of nuclear receptor interaction protein (NRIP)

Nuclear receptor interaction protein (NRIP), also named DCAF6 and IQWD1 was previously identified by our lab in 2005. NRIP was isolated by using the C-terminal of AR (androgen receptor) as the bait to screen a human HeLa cDNA library in a yeast two-hybrid screening assay (Tsai, Lee et al. 2005). Full-length NRIP cDNA contains 2583 base pairs of open reading frame that encodes a protein with 860 amino acids. It has seven WD-40 repeats and one IQ motif which was reported as an interaction domain of EF-hand motif containing protein (Chang, Tsao et al. 2011; Tsai, Lee et al. 2005).We previously demonstrated that NRIP is an AR-interaction protein and as a transcriptional co-activator which mediates AR-driven gene expression (Tsai, Lee et al. 2005; Han, Lee et al. 2008). Besides, NRIP was found to interact with calmodulin (CaM) in the presence of calcium and then activates calcineurin phosphatase (CaN) activity (Chang, Tsao et al. 2011).

1.2 The role of NRIP in muscle.

CaM (calmodulin) is a calcium-binding messenger protein and plays an important role in muscle contraction. CaM binding to calcium can activate myosin light chain kinase (MLCK) which active form can phosphorylate light chain of myosin head and increase myosin ATPase activity. Active myosin forms cross-bridges with actin and further creates

muscle contraction (Ivan, Hazel et al. 1993). In our previous study, we found that the IQ motif of NRIP is responsible for the interaction with CaM in the presence of calcium (Chang, Tsao et al. 2011). The fact that NRIP binding to CaM implied that NRIP may involve in muscle contraction. To further investigate the role of NRIP in muscle, we generated conventional NRIP knockout mice of which NRIP is knockout in all tissues, and used it as our animal models for the follow-up experiments. We found that conventional NRIP KO mice have reduced muscle force and impaired adaptive exercise performance. Mechanism studies suggested that NRIP is involved in CaN-NFATc1 pathway and regulates CaMKII phosphorylation to stimulate slow myosin expression and to further mediate muscle contraction (Hsing-Hsung Chen thesis).

Muscular dystrophy (MD) is a group of diseases that characterized by progressive muscle wasting and weakness. There are several types of MD including Duchenne and Becker MD, congenital MD, distal MD, Emery-Dreifuss MD, facioscapulohumeral MD, limb-girdle MD, and so on (Emery 2002; Kevin 1995). Recent research indicated that the mRNA levels of NRIP (IQWD1) are down-regulated in dystrophy tissues of limb-girdle muscular dystrophy (LGMD) patients in comparison to normal tissues of the same LGMD patients (Zhang, Ye et al. 2006). It implied that NRIP may be somehow involved in muscular dystrophy disease.

1.3 The close relationship between muscle and motor neuron

Muscle and motor neuron regulate each other precisely. Brain, the center of the nervous system, triggers muscle contraction and control its movements involving breathing which is essential for life by motor neuron (Kevin, Artem, Christopher 2010). Muscle movements are stimulated by neurotransmitters released by axon terminal of motor neuron. Specific receptors for neurotransmitters are located on plasma membrane of muscle fibers to receive the nervous stimuli and the activated signal transduction can further trigger the contraction of muscle fiber.

1.3.1 The properties of motor neuron.

A motor neuron is composed of a cell body, multiple branches of dendrites, and a single long axon. Dendrites receive chemical signals from the axon termini of other neurons, whereas the axon extending from the cell body creates action potential to stimulate the effector cell like skeleton muscle (Harvey, Arnold et al. 2000). Motor neurons are located in the lateral ventral horn of the spinal cord and in brainstem nuclei; and to innervate skeletal muscles, they send their axons out through ventral roots or cranial nerves, respectively (Jonathan, Jonathan 2010). A pool of motor neurons supplying a single muscle form a discrete longitudinal column which extends through several adjacent spinal segments in the lateral ventral horn (Stavroula, John 1983).
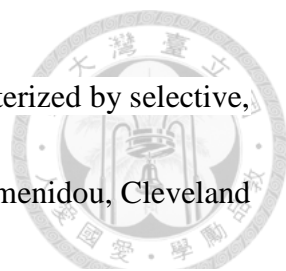
Motor neurons can be divided into two groups according to their targets: somatic

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motor neurons and visceral motor neurons. Somatic motor neurons which originate in the

central nervous system project their axons to the skeletal muscles and control movements

of skeletal muscle. Visceral motor neurons innervate cardiac muscles and smooth muscles

of the viscera and are responsible for regulating involuntary movements (Thaler, Koo et

al. 2004). Somatic motor neurons can be further subdivided into two subgroups: alpha

and gamma motor neurons. Alpha motor neurons (α-MNs) innervate skeletal muscles to

generate tension for skeletal movements. Gamma motor neurons (γ-MNs) innervate

muscle spindles to detect the change of length in muscle (Kevin, Artem, Christopher

2010). They can be distinguished by their cell size and specific expression markers. Cell

bodies of α-MNs (about $800\mu m^2$) are larger than that of γ-MNs (about $200\mu m^2$) (Andreas,

Julia et al. 2009). Both α-MNs and γ-MNs express choline acetyltransferase (ChAT)

which is the marker of cholinergic neurons (Barber, Phelps et al. 1984). The neuronal

DNA binding protein NeuN and the transcription factors Err3 can be used to distinguish

α-MNs and γ-MNs. α-MNs express NeuN only but not Err3, whereas γ-MNs express Err3

only but not NeuN (Andreas, Julia et al. 2009).

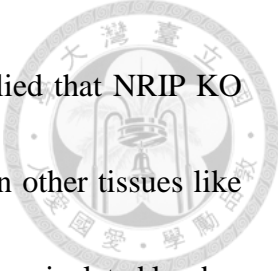### 1.3.2 Motor neuron degeneration disease.

Motor neuron diseases (MNDs) comprise a group of severe disorders characterized

by progressive degeneration of motor neurons. Degeneration of motor neurons may cause

muscle weakness and atrophy (Dion, Daoud, Rouleau 2009). Amyotrophic lateral

sclerosis (ALS) is the most common adult-onset MND that is characterized by selective, premature degeneration and death of motor neurons (Ilieva, Polymenidou, Cleveland 2009). Motor neuron degeneration initiates in mid-adult life and ALS patients usually die from respiratory failure within 2-3 years of symptom onset (Renton, Chiò, Traynor, 2014). About 90% of ALS is classified as sporadic, whereas the remaining 10% of cases are considered familial. Familial ALS is inherited in a dominant manner and some of these are linked to missense mutations of SOD1 (Cu/Zn superoxide dismutase type-1) (Sau, De Biasi et al. 2007; Saccon, Bunton-Stasyshyn et al. 2013). Another common MND called spinal muscular atrophy (SMA) is an autosomal recessive disease caused by deletion or mutation of the survival motor neuron 1 (*SMN1*). It is characterized by degeneration of spinal cord motor neuron, atrophy of skeletal muscles and muscle weakness (Zhang, Pinto et al. 2013; Lunn, Wang 2008). Common syndromes shared by both ALS and SMA patients are muscle atrophy and weakness resulted from degeneration of motor neurons.

1.4 The role of NRIP in motor neuron.

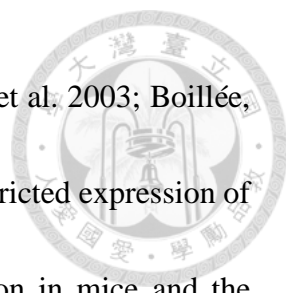In our previous study, we confirmed that conventional NRIP KO mice have reduced muscle force from *in vitro* muscle force experiments. It indicates that NRIP knockout can directly affect the intensity of muscle force. Besides, we found that conventional NRIP KO mice have impaired adaptive exercise performance from rotarod test. Rotarod test can be used to evaluate motor coordination, learning and cardiopulmonary endurance of

rodents (Shiotsuki, Yoshimi et al. 2010). Therefore, our results implied that NRIP KO

may not only cause abnormal physiology in muscle tissue but also in other tissues like

motor neuron. To further investigate the role of NRIP in motor neuron, we isolated lumbar

spinal cords of mice and measured the number of α-MNs in WT and conventional NRIP

KO mice. We found the loss of α-MNs in conventional NRIP KO mice. Moreover, gliosis

were observed in NRIP KO mice but not in WT mice. Those results indicate that NRIP

may also play an important role in motor neuron survival and maintaining (Yuan-Chun

Huang thesis).

1.5 Muscle-derived factors regulating motor neuron survival and maintaining.
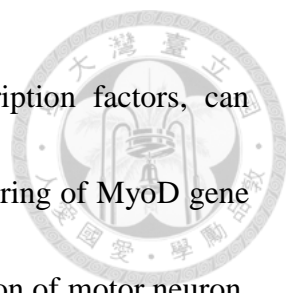
Motor neuron survival and maintaining are determined not only by itself but its

peripheral tissues or cells like muscles, astrocytes and microglia. Over the past 15 years,

scientists trended to focus on the importance of non-neuronal cells in maintaining neuron

function (Cortes, Ling et al. 2014). For instance, muscle pathology may contribute to the

Kennedy disease phenotype through both cell autonomous and non-autonomous

mechanisms (Yu, Dadgar et al. 2006). Previous research indicate that muscle-restricted

expression of mutant AR accounts for systemic and neuromuscular phenotypes in

Kennedy disease (also named spinal and bulbar muscular atrophy, SBMA) (Cortes, Ling

et al. 2014). Moreover, in ALS models, studies in several transgenic mouse models have

demonstrated that the mutant SOD1 protein acting within non-neuronal cells such as

microglia and muscles is toxic to motor neurons (Clement, Nguyen et al. 2003; Boillée, Yamanaka et al. 2006). These results help to explain why neuron-restricted expression of mutant SOD1 was insufficient to induce motor neuron degeneration in mice and the importance of non-neuronal cells in motor neuron survival (Lino, Schneide, Caroni 2002; Pramatarova, Laganière et al. 2001).

Neurotrophins, hormones or growth factors secreted by peripheral cells of MNs can regulate their survival, development, function and plasticity (Huang, Reichardt 2001). In the case of muscle-derived factors, there were many references providing evidences that these muscle-secreting or -expressing factors may regulate motor neuron survival and maintaining. For instance, muscle-derived neurotrophin-4 (NT-4) was previously reported as a neurotrophic signal for growth and re-innervation of adult motor neurons (Funakoshi, Belluardo et al. 1995). Insulin-growth factor 1 (IGF-1) is a hormone and a recent study has shown that muscle expression of IGF-1 can protect motor neuron from degeneration in ALS mice model (Dobrowolny, Giacinti et al. 2005). Bone morphogenetic proteins (BMP) belong to TGF-β family. BMP4 expressed by Schwann cells and skeletal muscle fibers was proved that it may mediate the survival of motor neurons (Chou, Lai et al. 2013).
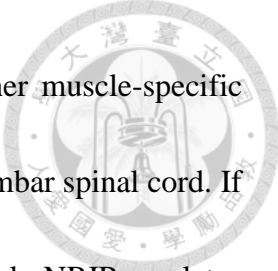
Except for those factors secreted by muscle, some non-secreting factors expressed by muscle can also regulate motor neuron survival and maintaining. Recent research have

suggested that MyoD and myogenin, both are myogenic transcription factors, can enhance or inhibit motor neuron degeneration, respectively. Transferring of MyoD gene into muscle can decrease motor neuron survival, enhance degeneration of motor neuron, and accelerate muscle denervation in ALS mice model. On the other hand, myogenin gene transfer can support motor neuron survival and maintain muscle innervation in ALS mouse model (Park, Franciosi, Leavitt 2013; Kablar, Belliveau 2005). Moreover, transgenic mice of which human SOD1 gene express in skeletal muscle display muscle pathology and neurologic phenotype consistent with ALS. Their results indicate that mutant SOD1 gene expressed by muscle can cause motor neuron degeneration (Wong, Martin 2010). From results of these previous research mentioned above, the muscle-derived factors may play crucial roles in motor neuron survival and maintaining.

1.6 Aim of this study

According to previous description, we found conventional NRIP KO mice have reduced muscle force, impaired adaptive exercise performance and the loss of motor neurons in spinal cord. Although we have confirmed that NRIP knockout could directly affect muscle function, the mechanism that NRIP causes the loss of motor neurons in NRIP KO mice is still unclear. Muscle and motor neuron regulate each other precisely. Previous studies also imply that muscle-expressing factors may regulate motor neuron survival and maintaining. We hypothesized that muscle-expressing NRIP may play a role

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in motor neurons. Thus, we were interested in investigating whether muscle-specific expression of NRIP could mediate the survival of motor neuron in lumbar spinal cord. If it is true, we will further try to study the mechanism by which muscle NRIP regulates motor neurons survival. The following are five aims of this thesis.

The aims of this study are as following:
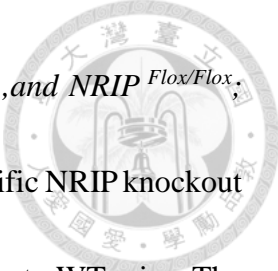
1. **To generate the muscle-specific NRIP knockout (cKO) mice as our experimental models.**

2. **To measure the number and size of α-MN in lumbar spinal cord of WT and cKO mice.**

3. **To investigate whether cKO mice have abnormal neuromuscular junction (NMJ) and axonal innervation compared with WT mice.**

4. **To study whether cKO mice have abnormal phenotype and impaired behavior performance.**

5. **To select muscle-derived candidate factors which may mediate motor neuron survival.**

Chapter 2 MATERIALS AND METHODS

2.1 Generation of muscle-specific NRIP knockout mice

Mouse NRIP genomic DNA (bMQ134h07) was obtained by screening a BAC library (Geneservice, http://www.geneservice.co.uk/) derived from the 129 mouse strain. This 19.2 kb mouse genomic DNA fragment was inserted at the NotI–SpeI site of L253, in which the MC1-TK (thymidine kinase) gene served as a negative selection marker. The resulting construct was used as the backbone for subsequent insertion of a loxP sequence from pL452 into intron 2 and of a neo cassette with two flanked loxP sequence from pL452 into intron 1. The final targeting construct contained a homologous short 5' arm of 4.3 kb and long 3' arm of 10.6 kb. The targeting vector was linearized by DNA digestion with NotI (unique site) and electroporated into embryonic stem (ES) cells, and then G418-resistant clones were selected. The ES cells containing the floxed NRIP allele were injected into blastocytes of C57BL/6JNarl mice and then implanted into pseudo-pregnant foster mothers (Chen, Tsai et al. 2012). The chimeric offspring was back-crossed to the C57BL/6JNarl mice (N $\geqq$ 10 generations) to generate the NRIP-LoxP heterozygous (*NRIP* $^{Flox/+}$) mice. To obtain muscle-specific knockout animals, *NRIP* $^{Flox/+}$ mice were crossed with MCK (muscle creatine kinase)-Cre heterozygous mice (Brüning, Michael et al. 1998) and then the *NRIP* $^{Flox/+}$; *MCK-Cre* $^+$ offsprings were crossed to each other resulting to six different genotypes: *NRIP* $^{+/+}$; *MCK-Cre* $^+$, *NRIP* $^{Flox/+}$;*MCK-Cre* $^+$, *NRIP*

Flox/Flox; *MCK-Cre* ⁺ , *NRIP* ^{+/+}; *MCK-Cre* ⁻, *NRIP* ^{Flox/+}; *MCK-Cre* ⁻ ,and *NRIP* ^{Flox/Flox};

MCK-Cre ⁻. Only *NRIP* ^{Flox/Flox}; *MCK-Cre* ⁺ mice were the muscle-specific NRIP knockout

mice (named conditional KO mice, cKO mice), whereas others belong to WT mice. The

genotypes of WT and cKO mice used for performing experiments are *NRIP* ^{Flox/Flox}; *MCK-*

Cre ⁻ and *NRIP* ^{Flox/Flox}; *MCK-Cre* ⁺, respectively. Routine genotyping was performed by

lysing tail samples by DirectPCR lysing reagent (VIAGEN) and then executing PCR

reaction. For WT and floxed NRIP genotyping, GU and JD primers were used. For cre

recombinase genotyping, four oIMR primers were used. The sequences of these primers

are shown below. The PCR reaction condition was 95℃ for 10 min; 35 cycles of 95℃

for 2 min, 60℃ for 45 sec and 72℃ for 1 min; and 72℃ for 10 min.

Primers for WT and floxed NRIP genotyping

NRIP30614GU: 5'-TTCGCGGCCGCTCTGTGAAGCTTACTTTCATTT-3'

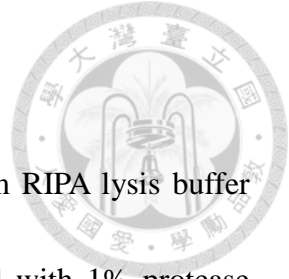NRIP33255JD: 5'-CTTGTCGACATTCTTATCAGCTACACTAG-3'

Primers for *Cre recombinase* genotyping

oIMR1085: 5'-GTGAAACAGCATTGCTGTCACTT-3'

oIMR6754: 5'-TAAGTCTGAACCCGGTCTGC-3'

oIMR8744: 5'-CAAATGTTGCTTGTCTGGTG-3'

oIMR8745: 5'-GTCAGTCGAGTGCACAGTTT-3'
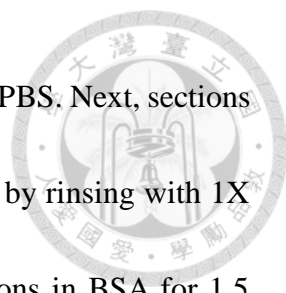
2.2 Western blot analysis

Various tissues were isolated and proteins were extracted from RIPA lysis buffer [150mM NaCl, 0.5% DOC, 1% NR40, 1.5M Tris 50mM (ph8.8)] with 1% protease inhibitor, DTT and PMSF. After centrifugation at the highest speed for 30 minutes, the supernatants were collected. Total proteins were then separated on 10% SDS-PAGE, and then transferred to PVDF membrane. Anti-NRIP antibody (Novas, 1:2500 dilution) was used to detect NRIP expression in various tissues and proteins were finally visualized by ELC Western blotting detecting system (Amersham Bioscience).

2.3 Mice spinal cord isolation and cryostat section preparation

To conduct spinal cord isolation, mice were anesthetized with 2.5% Avertin and sacrificed by heart excision. Then remove needless parts of mice and carefully retain the vertebra part. Poke an injector into the end of vertebra and flush out whole spinal cord by pumping PBS into the terminal of vertebra. The lumbar segment of spinal cord was quickly trimmed and then embedded into an O.C.T block. The block was placed in -20℃ refrigerator for one day and stored in -80℃ refrigerator afterwards. The O.C.T block was later cross-sectioned in 30 μm thickness by cryostat microtome.
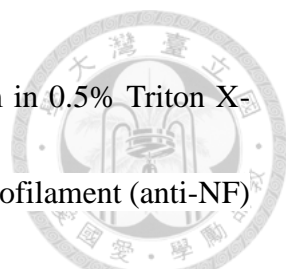
2.4 Immunofluorescence assay

Cryostat sections of 30 μm thickness were washed by 1X phosphate buffered saline (PBS) to purge O.C.T and then fixed in 4% paraformaldehyde (PFA) for 5 minutes.

Washing sections were done three times for 10 minutes each with 1X PBS. Next, sections were permeabilized in 1% Triton X-100 for 3 minutes and followed by rinsing with 1X PBS three times for 10 minutes each. Subsequently, blocking sections in BSA for 1.5 hours at room temperature and sections were stained with the primary antibody diluted by 1X PBS as following: anti-NeuN Ab (rabbit-monoclonal, Millipore, 1: 500 dilution) and anti-ChAT Ab (goat-monoclonal, Millipore, 1:250 dilution). Sections were incubated with primary antibodies overnight at 4℃. After primary antibodies incubation, sections were washed three times with 1X PBS for 10 minutes each and stained with secondary antibodies (Cy3-conjugated goat anti-rabbit and 488-conjugated goat anti-goat, 1: 500 dilution, Jackson ImmunoResearch Laboratories) for 1 hour in dark at room temperature. Next, washing sections with 1X PBS three times for 10 minutes each in dark and finally mounting it with DAPI Fluoromount-G (SouthernBiotech). The sections were later visualized and took pictures by Lecia TSC SP5 confocal microscope (Lecia Microsystems, Wetzlar, Germany) with 40X oil-immersion objective lens.

2.5 Whole-mounting staining of neuromuscular junction

Intact dissection of the soleus from tissue was fixed in 2% PFA for 30 minutes at room temperature, and teased apart using forceps followed by rinsing with 1X PBS three times for 30 minutes each. Soleus was incubated with 0.1M glycine in 1X PBS for 1 hour and then washed three times with 0.5% Triton X-100/PBS for 30 minutes each. Next,
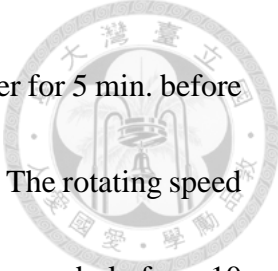
blocking soleus in blocking buffer (2.5% BSA and 5% horse serum in 0.5% Triton X-100/PBS) overnight at 4℃. Soleus was then incubated with anti-neurofilament (anti-NF) primary antibodies (rabbit-monoclonal, Abcam, 1: 500 dilution) in the blocking buffer overnight at 4℃ and followed by rinsing with 0.5% Triton X-100/PBS three times for 1 hour each. Subsequently, soleus was incubated with secondary antibodies (488-conjugated goat anti-rabbit, 1:1000 dilution) and Alexa-594-conjugated α-bungarotoxin (α-BTX, Life technologies, 1:1000 dilution) for at least 2 hours in dark at room temperature and followed by washing with 0.5% Triton X-100/PBS three times for 1 hour each. Finally, rising soleus once with 1X PBS and mounting it with DAPI Fluoromount-G. The whole-mounting sections were later visualized and took pictures by Lecia TSC SP5 confocal microscope with 40X oil-immersion objective lens.

2.6 Behavior test

In grip force assay, three WT mice and five cKO mice were tested when they were 12-week-old. Mice were first hold by their tails so that their forepaws could grasp a wire grid. Then the mice were gently pulled backward by the tail until they released the grid. The peak force applied by the forelimbs of the mouse was recorded in gram (g). Each mouse was tested three times, and the average value was used for statistical analysis (Matsuo, Tanda et al. 2009).

In rotarod assay, mice were placed at the 3-cm diameter cylinder of the rotarod

apparatus (Singa RT-01) and ran with specific rotating speed of cylinder for 5 min. before

starting of testing phase, three times of training phase were performed. The rotating speed

of each training phase is 5 r.p.m. In testing phase, rotating speed increase orderly from 10

r.p.m., 15 r.p.m., to 20 r.p.m. Latency time of mice under these three different speeds

would be measured and used for statistical analysis. The 5 min break time between each

measuring interval was provided in both training phase and testing phase.

2.7 Tissue RNA extraction and Real-Time quantitative PCR (RT-qPCR)

Muscle tissues were harvested, placed into 1.5ml eppendorf and lysed with 1ml

Trizol (Ambion). Adding 200 μl chloroform per 1ml Trizol solution and inverting

eppendorf up and down for 10 times by hand. The sample was incubated at room

temperature for 3 minutes and then centrifuged at 12,000g for 15 minutes at 4℃.

Following centrifugation, transferring the upper colorless aqueous phase to another

eppendorf with 500 μl isopropanol to precipitate RNA and inverting eppendorf up and

down for 10 times by hand. The sample was incubated at room temperature for 10 minutes

and then centrifuged at 12,000g for 5 minutes at 4℃. Removing the supernatant and using

1ml 75% ethanol to wash pellet followed by centrifugation at 7,600g for 5 minutes at 4℃.

Subsequently, removing the supernatant and air-drying the RNA pellet. RNA pellet was

dissolved by RNase-free DEPC-treated water and then measuring the concentration of

total RNA. 2 μg of RNA were reverse transcribed to cDNA using Superscript III reverse

transcriptase (Invitrogen). RT-qPCR was performed by using 5X HOT FIREPol®EvaGreen®qPCR Mix Plus (Solis BioDyne) on ABI7500 (Applied Biosystems).
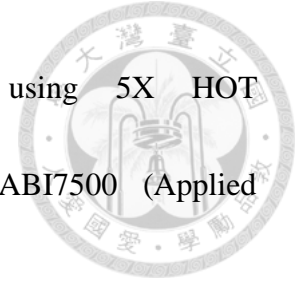
The following primers were used in RT-qPCR:

GAPDH-Fwd: 5'-CATGGCCTTCCGTGTTCCT-3'

GAPDH-Rev: 5'-GCGGCACGTCAGATCCA-3'

NT4-Fwd: 5'-AGCGTTGCCTAGGAATACAGC-3'

NT4-Rev: 5'-GGTCATGTTGGATGGGAGGTATC-3'

IGF1-Fwd: 5'-GTGGACCGAGGGGCTTTTACTTC-3'

IGF1-Rev: 5'-TTTGCAGCTTCGTTTTCTTGTTTG-3'

IGF2-Fwd: 5'-CGCTTCAGTTTGTCTGTTCGG-3'

IGF2-Rev: 5'-TGGGTGGTAACACGATCAGG-3'

IGFBP4-Fwd: 5'-CCATCCAGGAAAGCCTGCAG-3'

IGFBP4-Rev: 5'-TGGAAGTTGCCGTTGCGGTCACAG-3'

IGFBP6-Fwd: 5'-GCTCCAGACTGAGGTCTTCC-3'

IGFBP6-Rev: 5'-GAACGACACTGCTGCTTGC-3'

BMP4-Fwd: 5'-TCGCCATTCACTATACGTGGACTT-3'

BMP4-Rev: 5'-CACAACAGGCCTTAGGGATACTAGA-3'

Myogenin-Fwd: 5'-GCTGTATGAAACATCCCCCTA-3'

Myogenin-Rev: 5'-CGCTGTGGGAGTTGCATT-3'

AChRε-Fwd: 5'-TTCGCTCCCAGACCTACAATG-3'

AChRε-Rev: 5'-TCGTCATCCACGGCAAAGA-3'

NRIP-Fwd: 5'-TGGAAGAGCTGGATACTTTGAACA-3'

NRIP-Rev: 5'-AGTTGCGGTGGCCCTTATAAA-3'

2.8 C2C12 cell culture

C2C12 cells were cultured in DMEM (Gibco) supplemented with 10% FBS. For myoblast differentiation experiments, the growth medium (GM) was replaced with differentiation medium (DM), which comprised DMEM supplemented with 5% horse serum to induce myogenic differentiation. C2C12 myoblasts were grown to 80% confluence and then infected with the virus indicated at a dose of 10 MOI for 18 h in GM before differentiation. At the time indicated after differentiation, the cells were harvested and total RNA were extracted for the following RT-qPCR reaction.

2.9 Statistical analysis

Statistics was all calculated by Microsoft Excel 2013 software. Data were reported as the mean±SEM. All p values were determined by two-tailed student's t test for comparisons between 2 independent groups. A p value of less than 0.05 was considered

statistically significant difference. *p<0.05, **p<0.01, and ***p<0.001.

2.10 Study approval

All animal experiments were reviewed and approved by Institutional Animal Care

and Use Committee (IACUC) at the College of Medicine, National Taiwan University.

All experimental mice were housed in the animal center under a 12 h light/dark cycle with

free access to food and water.

Chapter 3 RESULTS

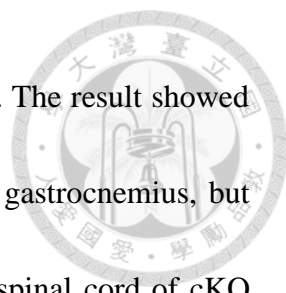## 3.1 Generation of muscle-specific NRIP knockout (cKO) mice.

We previously demonstrated that motor neuron loss was observed in spinal cord of conventional NRIP KO mice in which NRIP gene was knockout in all tissues (Yuan-Chun Huang thesis). Recently, several reports illustrate that skeletal muscle is a primary site of pathogenesis in ALS that causes MN degeneration (Wong, Martin 2010; Dobrowolny, Giacinti et al. 2005; Krakora, Macrander, Suzuki 2012). Thus, to study the role of muscle-derived NRIP in motor neuron, we generated muscle-specific NRIP knockout (cKO) mice by crossing mice possessing two floxed-NRIP alleles (*NRIP^Flox/Flox*) to MCK-Cre mice. Muscle creatine kinase (MCK) is expressed at high levels only in skeletal and cardiac muscle tissues. The promoter activity of MCK is 10^4-fold higher in skeletal muscle than in other non-muscle tissues such as kidney, liver, and spleen. Expression in cardiac muscle was also greater than in these non-muscle tissues by 2 to 3 orders of magnitude (Johnson, Wold, Hauschka 1989). Thus, MCK-Cre mice expressed the cre recombinase regulated under control of the muscle-specific MCK promoter only in muscle tissue. Schematic illustration of the genomic structure of floxed-NRIP allele was shown in Figure 1A. Floxed NRIP allele containing a neo cassette with two flanked loxP sequence in intron 1 and a loxP sequence in intron 2 was designed for NRIP deletion. The deletion of NRIP exon 2 caused a frame shift mutation that resulted in premature

termination. Breeding of *NRIP^{Flox/Flox}* mice with MCK-Cre mice resulted in offspring in which NRIP gene was specifically knockout in muscle tissue but expressed normally in other tissues.

PCR reaction was performed to check the genotype of mice. The genotyping strategy was to check whether mice possess LoxP sequence and Cre recombinase. For loxP sequence genotyping, GU and JD primers shown in Figure 1A were used. There were three results of loxP sequence genotyping: NRIP WT (*NRIP^{+/+}*), NRIP-LoxP homozygous (*NRIP^{Flox/Flox}*), and NRIP-loxP heterozygous (*NRIP^{Flox/+}*). The results of loxP sequence genotyping are shown in Figure 1B (up). The band with smaller size (about 680 bp) represented WT NRIP without loxP sequence and the bigger one (about 720 bp) represented NRIP containing loxP sequence. The genotyping result of *NRIP^{Flox/+}* mice possessing one WT allele and one floxed allele existed both smaller and bigger size of bands. On the other hand, the genotyping results for Cre recombinase were shown in Figure 1B (down). The band with smaller size (about 200 bp) was used as internal positive control. The genotyping result of Cre^+ mice existed both smaller and bigger size (about 450 bp) of bands. The *NRIP^{Flox/Flox};*Cre^+ mice were the cKO mice used in later experiments.

To confirm whether NRIP expressed specifically in muscle tissue, protein lysates extracted from various tissues including brain, liver, testis, spinal cord, heart, soleus and
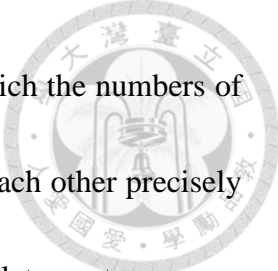
gastrocnemius of WT and cKO mice were analyzed by western blot. The result showed that NRIP is only knockout in muscle tissue like heart, soleus and gastrocnemius, but express normally in other tissues including brain, liver, testis, and spinal cord of cKO mice (Fig. 1C).
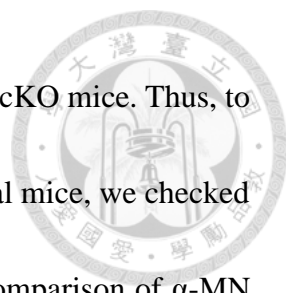
From Hsing-Hsung Chen thesis, we found that cKO mice display muscle pathology such as decreased number of slow myofibers, decreased expression of slow myosin, worse oxidative activity of slow myofibers and decreased expression of active NFATc1 regulated by NRIP. These phenomenon were also observed in conventional NRIP KO mice. In conclusion, we confirmed that the similar muscle pathology is shared by cKO mice and conventional KO mice.

3.2 The loss of motor neurons in muscle-specific NRIP knockout mice.

We have previously shown that 12-16 weeks old conventional NRIP KO mice possess deficient and less α-MNs in comparison to aged-matched WT mice. Besides, gliosis was also observed in spinal cord of 12-16 weeks old conventional NRIP KO mice (Yuan-Chun Huang thesis). It indicates that NRIP may play an important role in MN survival and maintaining. On the other hand, no statistically difference was observed between WT and conventional NRIP KO mice at their early ages (6-8 weeks old). We speculated that the loss of MN in KO mice was not development-related (Yuan-Chun Huang thesis). To study what mechanism by which NRIP knockout induces MN loss, the

following experiments were focused on 12-16 weeks old mice in which the numbers of

MN may be mediated by NRIP. Muscle and motor neuron regulate each other precisely

and researches suggested that muscle-expressing factors may regulate motor neuron

survival and maintaining mentioned in INTRODUCTION section. To further investigate

whether muscle-derived NRIP mediates motor neuron survival, we used muscle-specific

NRIP KO (cKO) mice as our experimental models and observed whether cKO mice have

deficient and less α-MNs in comparison to WT mice. Spinal cord was isolated and lumbar

sections were cross-sectioned in 30 uM thickness by cryostat microtome. Every fourth

cryostat section was used to perform immunofluorescence assay and count the numbers

of motor neuron (20 sections total from each animal). Anti-ChAT antibody and anti-NeuN

antibody were used to detect α-MNs. ChAT (choline acetyltransferase) is the marker of

cholinergic neuron and NeuN is the marker of α-MNs (Andreas, Julia et al. 2009). The

large cells co-expressing ChAT and NeuN were the α-MNs and were labeled in yellow

(Fig. 2A, arrow). Images were taken by confocal microscopy and were used to count the

number of α-MNs in WT and cKO mice spinal cord. Statistic data indicated that WT mice

had about 21 α-MNs per section and cKO mice had about 13 α-MNs per section (Fig. 2B).

There is significant difference in α-MN number between WT and cKO mice. The results

reveal that muscle-derived NRIP might mediate the survival and maintaining of MNs in

spinal cord.

We observed MN loss in both conventional NRIP KO mice and cKO mice. Thus, to compare the degree of MN loss between conventional and conditional mice, we checked the data of conventional mice from Yuan-Chung. Statistic data of comparison of α-MN number between conventional mice and conditional mice indicated that there was no significant difference in the number of α-MNs between conventional WT (WT') and conditional WT mice. There were about 20 α-MNs per section in both WT' and WT mice. However, the number of α-MNs in conventional KO (KO') mice was lower than that in cKO mice. Conventional KO mice had approximately 9 α-MNs per section whereas cKO mice had approximately 13 α-MNs per section. To eliminate the operative error from different people, we compare the percentage of survived α-MNs between KO' mice and cKO mice. Statistic data showed that the proportion of α-MNs number in KO' mice is lower than 50% in WT' mice. However, the proportion of α-MNs number in cKO mice is about 60% in their control mice (Fig. 2C). There are significant difference in the percentage of survived α-MNs between KO' mice and cKO mice. It suggested that muscle effects are just the partial reasons which induce MN loss in conventional KO mice although muscle-specific NRIP knockout is sufficient to cause MN loss in spinal cord. The difference in MN number between KO' and cKO mice represented that NRIP knockout in other tissues or cells like MN itself may also affect its survival.

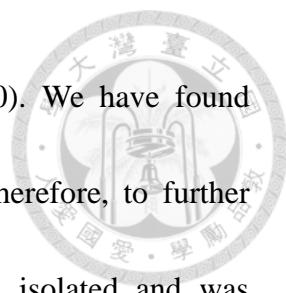Besides, we examined whether cKO mice have abnormal size of α-MNs. We

measured the area and perimeter of ventral horn α-MN. The results indicated that 12-16

weeks old cKO mice had smaller soma area and shorter perimeter of α-MN than the age-

matched WT mice (Fig. 2D and 2E).
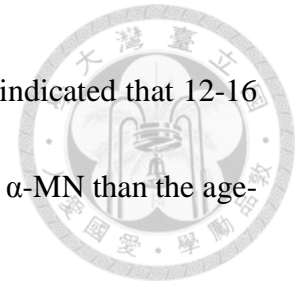
3.3 Muscle-specific NRIP KO mice have smaller NMJ area and lower axonal innervation percentage in NMJ.

The neuromuscular junction (NMJ) located between presynaptic axon of motor

neuron and postsynaptic membrane of muscle is a complex structure that mediates the

cross-talk between motor neurons and muscle fibers (Arnold, Gill et al. 2014; Sanes,

Lichtman 1999). It is at the NMJ that a motor neuron is able to transmit a signal to the

muscle fiber. To achieve efficient neuromuscular transmission, acetylcholine receptors

(AChRs) must be densely clustered on the postsynaptic muscle membrane of the NMJ.

The muscle-specific receptor tyrosine kinase (MuSK) and the motor neuron-derived agrin

which is a MuSK activator play essential roles in the formation and maintenance of NMJs

(Tezuka, Inoue et al. 2014). When motor neurons release agrin, AChRs aggregate to form

clusters in the membrane of muscle fibers (Reist, Werle, McMahan 1992). After

acetylcholine released from axon terminal of MN are recognized by AChR, an action

potential is generated; the concentration of calcium ion in muscle cells increases; and

finally muscle contraction is triggered (Berchtold, Brinkmeier, Müntener 2000).

Recent study indicates that muscle-specific expression of human SOD1 causes motor

neuron degeneration and NMJ abnormalities (Wong, Martin 2010). We have found

muscle-specific NRIP KO mice have smaller and less α-MNs. Therefore, to further

investigate whether cKO mice have abnormal NMJ, soleus was isolated and was

performed whole-mounting staining to observe the NMJ of mice. Alpha-Bungarotoxin

(α-BTX) , the venom of *Bungarus multicinctu*, is a highly selective ligand for

acetylcholine receptor (AChR) which form clusters in the postsynaptic membrane when

receiving neural stimuli (Valdez, Tapia et al. 2010). Fluorescently-tagged α-BTX was

used to label NMJ and anti-neurofilament (anti-NF) antibody was used to detect motor

axon. As shown in Figure 3A, NMJ structure was labeled in red, motor axons were labeled

in green and muscle nuclei were labeled in blue with DAPI staining. The results

demonstrated that 12-16 weeks old cKO mice had abnormal morphology of NMJ and

deficient axonal innervation in comparison to age-matched WT mice (Fig. 3A). Images

were taken by confocal microscopy and were used to measure the NMJ area and axonal

innervation percentage in NMJ of WT and cKO mice by ImageJ software. The area of

BTX-labeled AChR represented NMJ area; and the percentage of innervating axon area

overlapping with AChR in NMJ area meant axonal innervation percentage

(Borselli, Storrie et al. 2010; Sinha, Jang et al. 2014). Statistic data showed that the NMJ

area was about 410 um^2 in WT mice and was about 263 um^2 in cKO mice (Fig. 3B). The

percentage of axonal innervating area overlapping with AChR was about 17% in WT

mice and 10% in cKO mice (Fig.3C). Therefore, the results indicated that cKO mice have

significantly smaller NMJ area and lower axonal innervation percentage in NMJ in

comparison to WT mice.

3.4 Behavior test in WT and cKO mice.

Because we discovered that cKO mice have deficient MNs and abnormal NMJ in

comparison to WT mice, we next wondered whether cKO mice have abnormal phenotype

and impaired behavior performance. We measured the body weight of WT and cKO mice

when they were 12 weeks old. There was no significant difference in body weight

between 12-week-old WT and cKO mice (Fig. 4A). Next, to further confirm whether cKO

mice have weaker muscle force than WT mice, we performed grip force assay which is

used to assess forelimb grip strength (Matsuo, Tanda et al. 2009). Three WT mice and

five cKO mice were tested, and we found that cKO mice did not display deficient grip

force compared to WT mice (Fig. 4B). Besides, we performed the rotarod test which is

used to measure the association of NRIP expression with muscle function and motor

coordination. Both WT and cKO mice were placed on a rod rotating with increasing rate

from 10 r.p.m., 15 r.p.m., to 20 r.p.m.; and measured their riding time respectively at

different rate. The results showed that there was no significant difference between WT

and cKO mice under the condition of 10 r.p.m. and 15 r.p.m. However, cKO mice had

worse latency than WT mice under the condition of 20 r.p.m. (Fig. 4C). It indicates that
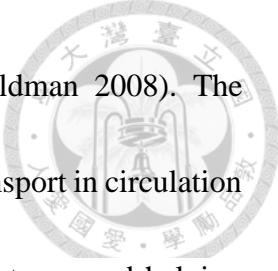
cKO mice display deficient motor function under severer environment.

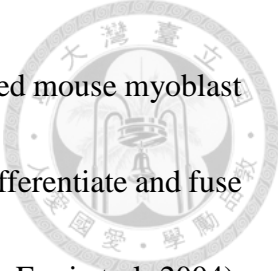**3.5 Selection of muscle-derived candidate factors which may affect motor neuron survival.**

The previous results in this thesis showed that muscle-specific NRIP knockout causes the loss of MNs, smaller NMJ size, and abnormal axonal innervation. Also, we found cKO mice have impaired adaptive exercise performance from rotarod test. That is an interesting phenomenon because NRIP expresses normally in spinal cord. These results implied that the lack of NRIP expression in muscle is sufficient to impair the normal functions of MN and NMJ. Many studies suggest that muscle-expressing factors may regulate motor neuron survival and maintaining mentioned in INTRODUCTION section. Thus, we next examined the specific factors which are expressed by muscle and can mediate MN survival and NMJ integrity.

Our strategy was to compare mRNA levels of various candidate genes in gastrocnemius or soleus muscle between WT and cKO mice. Those candidate factors we chose are NT4, IGF-1, IGF-2, IGFBP-4, IGFBP-6, BMP4, myogenin, and AChRε. NT-4 was previously reported as a neurotrophic signal for growth and re-innervation of adult motor neurons (Funakoshi, Belluardo et al. 1995). IGF-1 is a hormone and recently study showed that muscle expression of IGF-1 can protect motor neuron from degeneration in ALS mice model (Dobrowolny, Giacinti et al. 2005). Both IGF-1 and IGF-2 play a crucial

role in neurite outgrowth during development (Sullivan, Kim, Feldman 2008). The

functions of IGFBP (IGF binding protein) include regulating IGFs transport in circulation

or out of the vascular compartment, localizing IGFs to specific cell types and helping

IGFs to bind their receptors (Clemmons 1993). Recent study showed that IGFBP-6

expression is higher in ALS patients than in controls (Wilczak, Vos, Keyser 2003). BMP4

expressed by Schwann cells and skeletal muscle fibers was proved that it may mediate

the survival of motor neurons (Chou, Lai et al. 2013). Myogenin can support motor

neuron survival and maintain muscle innervation in ALS mouse model (Park, Franciosi,

Leavitt 2013; Kablar, Belliveau 2005). AChR is the receptor of acetylcholine which is a

kind of neurotransmitter. AChRε ablation has been reported to affect muscle contraction

and motor performance (Witzemann, Schwarz, et al. 1996).

To measure the mRNA levels of various candidate genes in gastrocnemius or soleus,

muscle tissue were isolated to extract tissue RNAs. Tissue cDNAs were obtained by

reverse-transcription reaction and mRNA levels were finally detected by RT-qPCR

reaction. In the result of gastrocnemius mRNA expression, only IGF2 mRNA levels in

gastrocnemius of cKO mice were significantly lower than that in WT mice (WT: n=6,

cKO: n=7) (Fig. 5A). However, in the result of soleus mRNA expression, only myogenin

mRNA levels in soleus of cKO mice were significantly lower than that in WT mice (WT:

n=4, cKO: n=4) (Fig. 5B). To double confirm whether NRIP regulate the expression of

IGF2 and myogenin, C2C12 cells were used. C2C12 is an immortalized mouse myoblast cell line. The cells readily proliferate in high-serum conditions, and differentiate and fuse in low-serum conditions (McMahon, Anderson et al. 1994; Burattini, Ferri et al. 2004). Differentiated C2C12 cells were infected with either Ad-shLuc or Ad-shNRIP. Small hairpin NRIP sequence (shNRIP) is a short RNA sequence that can target NRIP gene and then knockdown the expression of NRIP. Adenoviruses were used as a carrier to bring shRNA sequence into cells by infection. Ad-shLuc was used as controls. Three days after infection, total RNAs of cells were extracted and reverse-transcribed to cDNA. Then, mRNA levels of IGF2 and myogenin were measured by RT-qPCR reaction. The results showed that NRIP knockdown in C2C12 cells decreased myogenin mRNA levels compared with control. In contrast, IGF2 mRNA levels did not display apparent change in C2C12 cells in which NRIP gene is knockdown in comparison to controls (Fig.5C). These results imply that NRIP may regulate the expression of myogenin; and myogenin may be the muscle-derived factor that can cause the abnormal NMJ, impaired axonal innervation and the loss of MNs.

Chapter 4 DISCUSSION

In this study, we used muscle-specific NRIP KO mice as our experimental animal models to study the role of muscle-expressing NRIP in motor neuron. To obtain muscle-specific NRIP KO animals, mice with a floxed NRIP allele were crossed with animals transgenically expressing cre recombinase under the control of the muscle creatine kinase (MCK) promoter (Handschin, Choi et al. 2007; Chen, Tsai et al. 2012). NRIP is only knockout in muscle tissues like heart, soleus and gastrocnemius, but expressed normally in other tissues such as the spinal cord of cKO mice (Fig. 1C). In cKO mice, we found the loss of MNs in lumbar spinal cord compared to WT mice (Fig. 2B). Except for the decrease of MN numbers, both MN soma area and MN perimeter are smaller in cKO mice than in WT mice (Fig. 2D and 2E). Previous researches showed that muscle-derived factors like NT4, IGF-1, BMP4, and myogenin may protect MNs from degeneration in MN degeneration disease models mentioned in INTRODUCTION section. In ALS models, studies have demonstrated that mutant SOD1 gene expressed by muscle can directly contribute to muscular pathology and cause motor neuron degeneration (Wong, Martin 2010; Dobrowolny, Aucello et al. 2008). In addition, a recent study indicated that muscle-expressing mutant AR results in smaller MN soma area and perimeter in SBMA models (Cortes, Ling et al. 2014). Comparing our results with studies of other groups, we come to the similar conclusions that muscle-derived factors may play

a crucial role in motor neuron survival and maintaining.

We found significant MN loss in both conventional NRIP KO mice and cKO mice, NRIP expression is knockout in all tissues in the former mice and only knockout in muscle tissue in the later mice. It implies that muscle-specific NRIP knockout is sufficient to cause MN loss in lumbar spinal cord. To illustrate whether muscle-specific NRIP knockout is the only cause that induce MN loss, we compared MN number between conventional mice and conditional mice. Statistic data showed that there was no difference in MN number between two groups of WT controls. In contrast, MN number in conventional KO mice is significantly lower than that in cKO mice. Besides, there are significant difference in the percentage of survived α-MNs between KO' mice and cKO mice (Fig. 2C). The result suggested that the extent of MN injury in conventional KO mice is severer than in cKO mice. It also implied that muscle effects are just the partial reasons which induce MN loss in conventional KO mice whereas NRIP knockout in other tissues or cells like MN itself may also affect its survival. Just like what we knew: motor neuron survival and maintaining are determined not only by itself but by its peripheral tissues or cells like muscle, astrocytes and microglia (Huang, Reichardt 2001). For instance, previous study has shown that motor neuron-specific TDP-43 knockout mice display motor neuron loss in spinal cord (Wu, Cheng, Shen 2012). Thus, the role of motor

neuron-expressing NRIP in its survival is worth for further investigating. We are going to utilize motor neuron cell lines such as NG108-15 or NSC34 cells to study it.
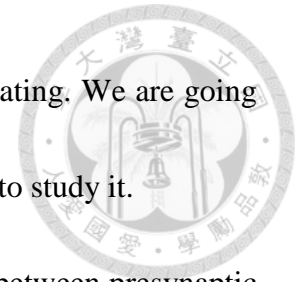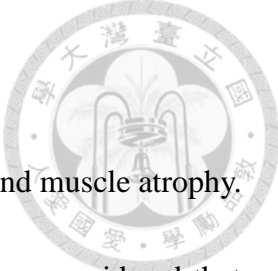
Neuromuscular junction (NMJ) is a complex structure located between presynaptic axon of motor neuron and postsynaptic membrane of muscle. In NMJ, neurotransmitters are released from axon terminal of motor neurons and recognized by specific receptors in muscle membrane to stimulate motor action. In this study, we observed MN loss in muscle-specific NRIP KO mice and concluded that muscle-expressing NRIP plays an important role in motor neuron survival. Recent study indicated that muscle-specific expression of human SOD1 causes both NMJ abnormalities and motor neuron degeneration in ALS models (Wong, Martin 2010). Evidences from animal models showed that NMJ degeneration is observed in the early stage of the ALS disease progression while the cell bodies in the spinal cord are mostly intact. Afterward, the "dying back" hypothesis was proposed. "Dying back" means a progressive distal to proximal degeneration and it is a common pattern seen in a wide variety of degenerative and toxic conditions of the central and peripheral nervous system. According to the "dying back" hypothesis, NMJ degeneration may actively cause the loss of MN (Dadon-Nachum, Melamed, Offen 2011; Krakora, Macrander, Suzuki 2012). Thus, we hypothesized that muscle-specific NRIP KO may somehow affect the normal function of NMJ so that it further causes MN degeneration. From our results, we indeed found that cKO mice have

abnormal morphology of NMJ (Fig. 3A). In muscle-specific expression of human SOD1 transgenic mice, NMJ structures are small, collapsed and condensed (Wong, Martin 2010). Compared with WT controls, we also discovered that NMJs are smaller and collapsed in cKO mice (Fig. 3A and 3B). Besides, we did not observe the phenomenon of severe denervation of motor axon. Almost all of the stained NMJs are innervated with motor axons. However, after calculating the percentage of innervating axon area overlapping with NMJ in NMJ area, we found that cKO mice has lower axonal innervation percentage than WT mice (Fig. 3C). Lower axonal innervation percentage may be an implication that axonal denervation will happen if we prolong our observation duration. There are a wide range of proteins that are highly concentrated in the postsynaptic membrane of muscle (Hoch 1999). Both AChR and MuSK (muscle-specific kinase) which is a kind of receptor tyrosine kinase are expressed in the postsynaptic membrane of muscle and are required for the formation and maintenance of the NMJ. AChR aggregation and clustering are one of crucial step in NMJ formation and are regulated by stimuli from neurons or signaling transduction from muscle cells. For instance, previous research showed that PGC-1α (peroxisome proliferator-activated receptor gamma coactivator 1-alpha) which is involved in regulation of energy metabolism in mitochondria can maintain NMJ integrity through mediating AChR clustering (Gouspillou, Picard et al. 2013). Therefore, it is worth for future investigation of whether NRIP regulates mitochondria function that

resulted in the effect of NMJ integrity.

Deficient MN and NMJ cause dysfunction of nerve conduction and muscle atrophy. Since MN loss and NMJ abnormalities are observed in cKO mice, we considered that cKO have impaired behavior performance. Rotarod test is used to evaluate motor coordination, learning and cardiopulmonary endurance of WT and cKO mice (Shiotsuki, Yoshimi et al. 2010). Results showed that cKO mice have worse latency than WT mice under the condition of 20 r.p.m. (Fig. 4C). Our previous study indicated that conventional NRIP KO mice display extremely poor scores in rotarod test under the condition of 10 r.p.m. In contrast, there is no significant difference between WT and cKO mice under the condition of 10 r.p.m. and 15 r.p.m. It indicates that cKO mice have lighter adaptive exercise performance than conventional KO mice and display deficient motor function under severer environment. We considered that these results are reasonable because conventional NRIP KO mice lack NRIP expression in all tissues whereas cKO mice only lack NRIP expression in muscle tissue. We infer that conventional NRIP KO mice display extremely poor performance in rotarod test because deficient NRIP expression in other tissues except for muscle may also play important roles in neuromuscular function. Many evidences reveal that specific genes expressed by motor neurons or peripheral nerve system can regulate motor neuron survival. For instance, motor neuron-specific TDP-43 knockout mice display motor neuron loss and poor performance in rotarod test (Wu,
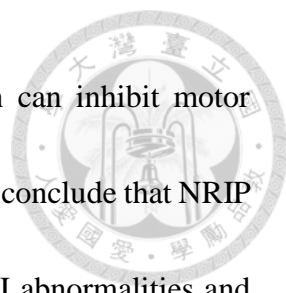
Cheng, Shen 2012). Progranulin secreted by motor neurons has also been proved that it can promote the survival of MNs (Ryan, Baranowski et al. 2009). Fibroblast growth factor-2 secreted by astrocytes is capable of increase motor neuron survival (Albrecht, Dahl et al. 2002). In summary, NRIP expressed in motor neurons or peripheral nerve system may also play crucial roles in neuromuscular function although muscle-specific NRIP knockout is sufficient to induce motor neuron degeneration.

Multiple capabilities of mice can be evaluated in rotarod test, but it cannot directly test the muscle force of mice. To investigate whether cKO mice have weaker muscle force in comparison to WT mice, we performed grip force assay by which forelimb grip strength of mice can be directly measured. From our previous study, we confirmed that conventional NRIP KO mice have reduced muscle force from *in vitro* muscle force experiments. We also illustrated that NRIP stimulates slow myosin expression and further mediates muscle contraction through involving in CaN-NFATc1 pathway and regulating CaMKII phosphorylation (Hsing-Hsung Chen thesis). Therefore, we predicted that cKO mice will have lower score in grip force assay than WT mice. However, results showed that cKO mice did not display deficient grip force in comparison to WT mice (Fig. 4B). In this experiment, only three WT mice and five cKO mice were tested. Maybe the animal models are not many enough to evaluate the significant difference between WT and cKO mice. In the future, we will increase the animal models or perform other analysis such as

ex vivo and in situ analysis of a single intact limb muscle and downhill treadmill exercise, all of them can be used to monitor murine skeleton muscle functions (Hakim, Li, Duan 2011).
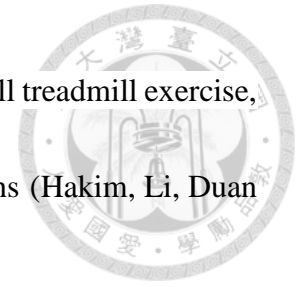
Our major finding in this study is that muscle-specific NRIP KO is sufficient to cause MN loss and NMJ abnormalities. It illustrates that muscle-restricted expression of NRIP plays an important role in regulating MN survival and NMJ integrity. To understand the mechanism that NRIP are involved in, we tried to find the candidate factors that are regulated by NRIP. In our results, we found that myogenin may be the muscle-derived factor which can cause the NMJ abnormalities and the loss of MNs. Myogenin mRNA expression is down-regulated in soleus tissue of cKO mice and C2C12 cells infected with Ad-shNRIP (Fig. 5B and 5C). In contrast, there are no statistical differences in myogenin mRNA levels of gastrocnemius tissue between WT and cKO mice (Fig. 5A). Gastrocnemius muscle is composed of approximately 50% slow and 50% fast muscle fibers whereas soleus muscle is composed of about 90% slow muscle fibers (Edgerton, Smith, Simpson 1975). Moreover, myogenin is a myogenic transcription factor predominantly expressed in adult slow muscles (Park, Franciosi, Leavitt 2013). We confirm that myogenin expression is regulated by NRIP in slow muscle fibers like soleus muscle. It is possible that we do not see the down-regulation of myogenin mRNA levels in gastrocnemius muscle of cKO mice because of its muscle constituents including fast

and slow muscle fibers. Recently, research suggest that myogenin can inhibit motor neuron degeneration (Park, Franciosi, Leavitt 2013). In this study, we conclude that NRIP KO in muscle decrease myogenin expression and further cause NMJ abnormalities and then extend to the loss of MN. In the future, we will demonstrate the detailed mechanism that NRIP regulates myogenin in muscle cell to influence the MN survival and NMJ integrity.

All experiments in this study are *in vivo* experiments using cKO animal models. To double confirm the phenomenon we observed in animal models, we will further perform *in vitro* experiments by a NRIP knockout system in C2C12 cells. The NRIP knockout system in C2C12 cells is generated by using CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) system. Designed guide-RNA targets to sequences near to start codon sequence of NRIP, double strand break (DSB) is then triggered by specific nuclease. Responded DNA repair mechanism such as NHEJ (non-homologous end joining) and homologous recombination result of approximately 30-60 base pairs deletion and premature termination of NRIP so that NRIP expression is depleted. Moreover, to investigate the muscle-motor neuron interaction, C2C12 cells and differentiated motor neuron cell line such as NG108-15 cells or NSC34 cells can be used because they are capable of forming functional neuromuscular synapses when co-cultured (Vianney, Spitsbergen 2011). The results from previous research show that muscle-

derived BMP4 can protect motor neuron from degeneration resulted from glutamate-induced toxicity in a NG108-15 cell-C2C12 cell co-culture system (Chou, Lai et al. 2013). Another study also shows that extracellular vesicles from C2C12 cells enhance cell survival and neurite outgrowth of NSC-34 cells (Madison, McGee et al. 2014). Their results imply that vesicles secreted from C2C12 cells or genes expressed by C2C12 cells are capable of increasing the survival of motor neuron cells such as NG108-15 cells or NSC34 cells in the co-culture system. In our previous results, we found muscle-specific NRIP knockout may regulate the survival of motor neurons in animal models. Therefore, we will further investigate whether C2C12 cells in which NRIP is knockout are capable of mediating the survival of motor neuron cells by using C2C12 cell-NSC-34 cell co-culture system.

Next, we will further confirm whether NRIP can rescue MN loss of cKO by developing adeno-associated virus (AAV)-mediated gene therapy to deliver NRIP into cKO mice. AAV is a small and nonenveloped virus that is used as a gene transfer vehicle. Previous studies indicated that gene transfer mediated by AAV has no toxicity and the triggered immune responses are relative mild (Samulski, Muzyczka, 2014). In this study, we found muscle-specific NRIP KO induces NMJ abnormalities and MN loss. We will inject AAV virus carrying NRIP sequence into muscle tissue of cKO mice. We hope NMJ abnormalities and MN loss can be rescued after gene therapy. The muscular pathology

and neural pathology observed from cKO mice are similar with those in muscular

dystrophy and ALS patients. Therefore, we expected that the gene therapy which AAV

carrying NRIP sequence can be used in treating the patients with muscular dystrophy such

as LGMD or motor neuron degeneration disease such as ALS to cure muscle atrophy,

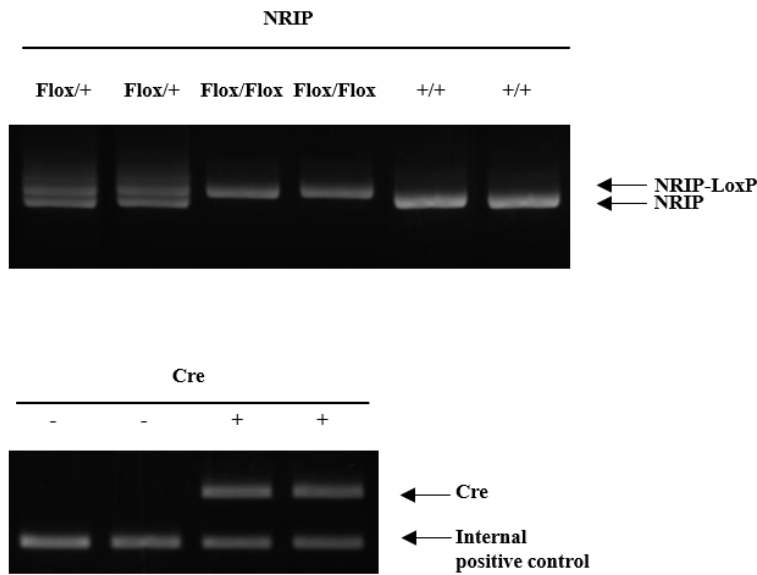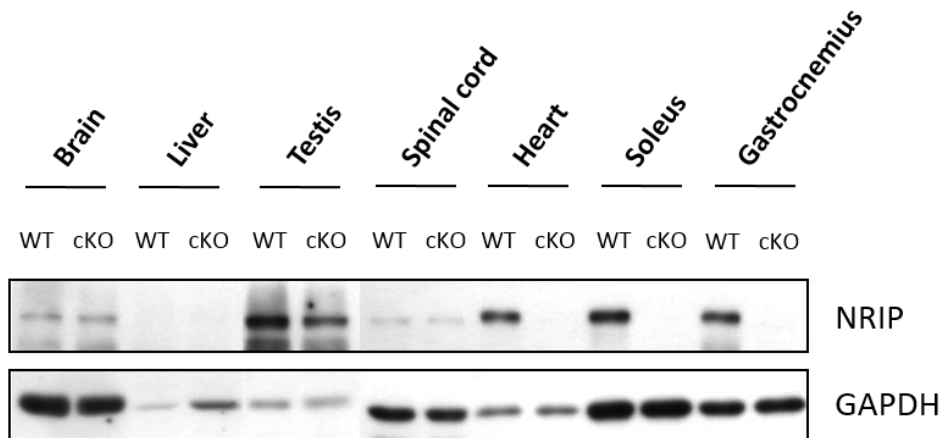NMJ collapsed and motor neuron degeneration.
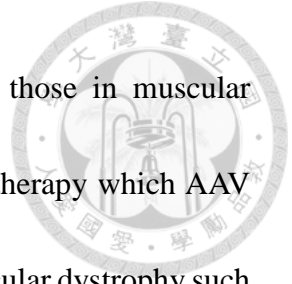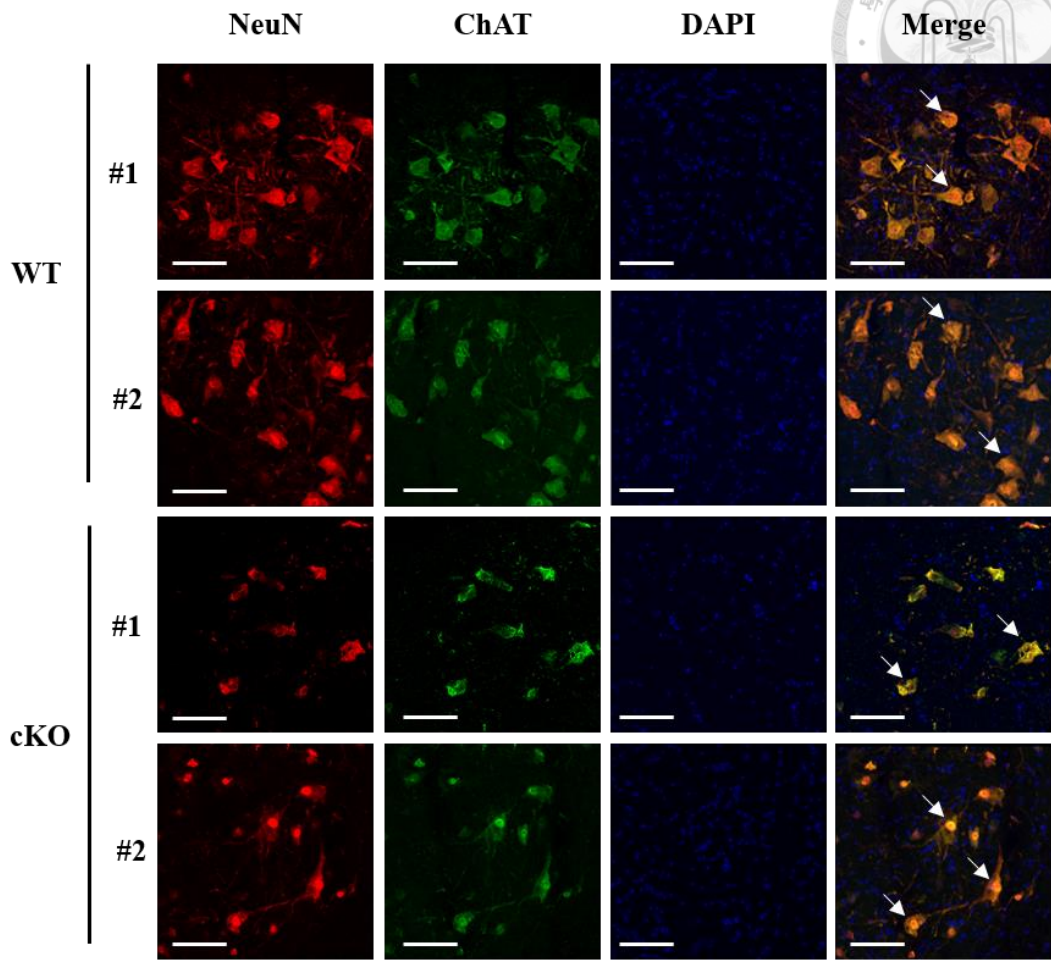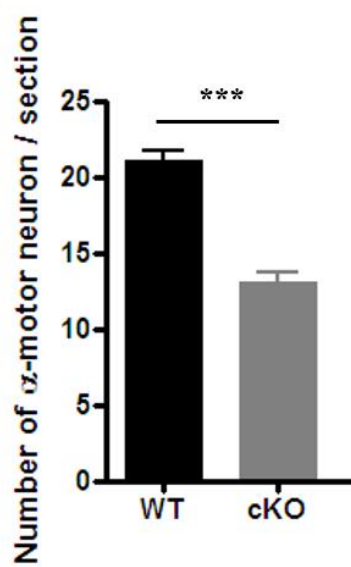
Chapter 5 FIGURES

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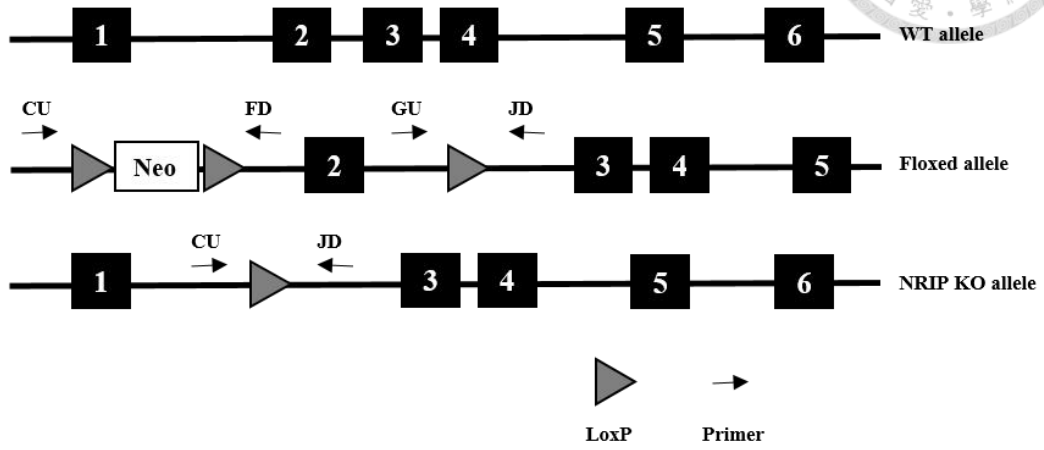
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Figure 1. Generation of muscle-specific NRIP knockout (cKO) mice. (A) Schematic illustration of the genomic structure of NRIP wild type, loxP-floxed NRIP and NRIP-deleted alleles. The loxP-floxed allele contains a neo cassette with two flanked loxP sequence in intron 1 and a loxP sequence in intron 2. NRIP exon 2 would be deleted by Cre recombinase driven by MCK promoter. (B) Genotyping by PCR. For WT and floxed NRIP genotyping (up), GU and JD primers are used. NRIP$^{Flox/+}$ represents that mice possess one WT allele and one floxed allele. NRIP$^{Flox/Flox}$ represents that mice possess two floxed alleles. NRIP$^{+/+}$ represents that mice possess two WT alleles. For genotyping of Cre recombinase (down), four IMR primers mentioned above are used. Mice possessing upper and lower bands are the cre-positive mice. In contrast, mice only possessing the lower band are the cre-negative mice. (C) Western blot analysis for expression of NRIP protein in various tissues from WT and cKO mice. The results show that NRIP is only knockout in muscle tissue like heart, soleus and gastrocnemius, but express normally in other tissue of cKO mice. GAPDH was used as internal control.

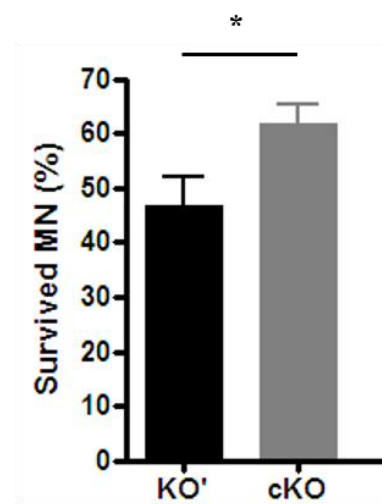**(A)**

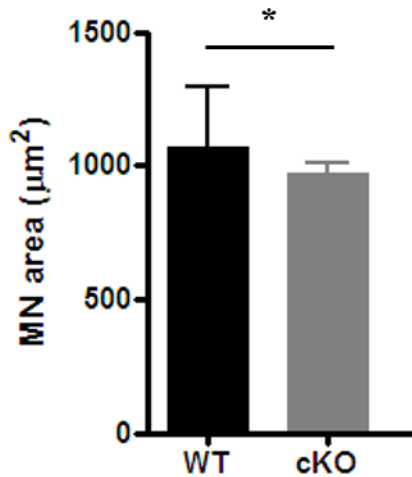| | NeuN | ChAT | DAPI | Merge |

WT #1, #2

cKO #1, #2

Scale bar: 100 μm

(B)

Number of α-motor neuron / section

***

WT, cKO

(C)

Survived MN (%)
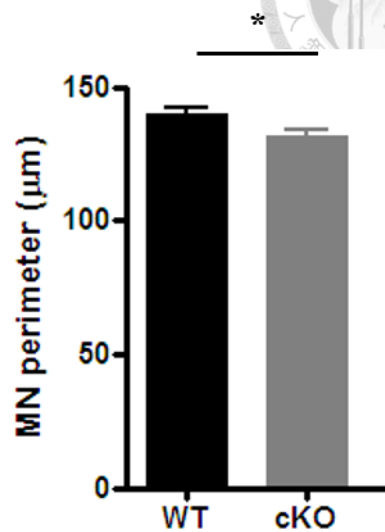
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KO', cKO

(D)

(E)

**Figure 2. The loss of motor neurons in muscle-specific NRIP knockout (cKO) mice.**
(A) Immuno-fluorescent analysis of NeuN (red), ChAT (green), and DAPI(blue) expression in lumbar spinal cord of 12-16 weeks old mice. In merged images, larger size of cells which co-express NeuN and ChAT were the α-motor neurons (α-MNs) (arrow). Images was taken by confocal microscopy. There are two groups of representative images from both WT and cKO lumbar spinal cord section. Scale bar: 100 um.   (B) Quantification of ventral horn α-MNs numbers per frozen section shown in panel (A). The result showed that 12-16 weeks old cKO mice exhibit α-MNs loss in comparison to age-matched WT controls (WT: n=5; cKO: n=5). ***$p<0.001$, student's t test. (C) Comparison of the percentage of survived α-MNs between conventional NRIP KO (KO') mice and cKO mice. The result showed that the proportion of α-MNs number in KO' mice is lower than 50% in WT' mice. However, the proportion of α-MNs number in cKO mice is about 60% in their control mice. There are significant difference in the percentage of survived α-MNs between KO' mice and cKO mice. *$p<0.05$, student's t test. (D) Quantification of the size of α-MNs in spinal cord of WT and cKO mice. The result showed that 12-16 weeks old cKO mice had smaller α-MNs than the aged-matched WT mice. *$p<0.05$, student's t test. (E) Quantification of MN perimeter in spinal cord of WT and cKO mice. The result showed that 12-16 weeks old cKO mice had shorter MN perimeter than the aged-matched WT mice. *$p<0.05$, student's t test.

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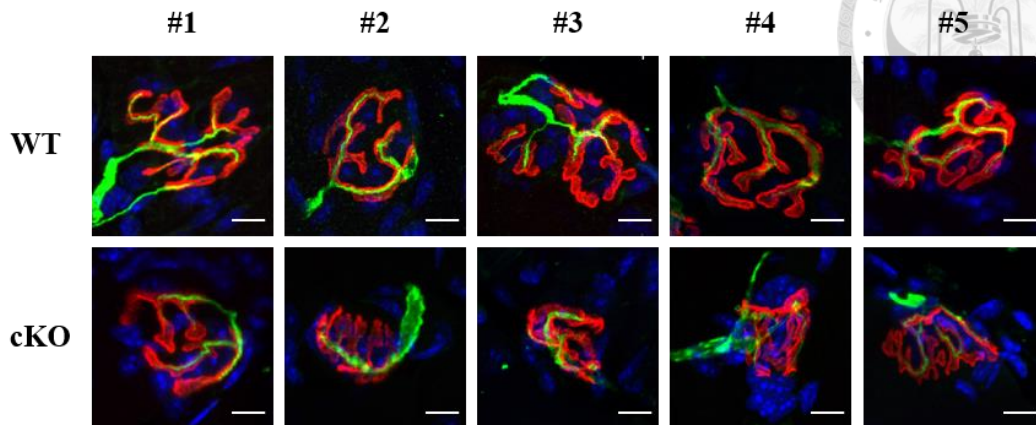
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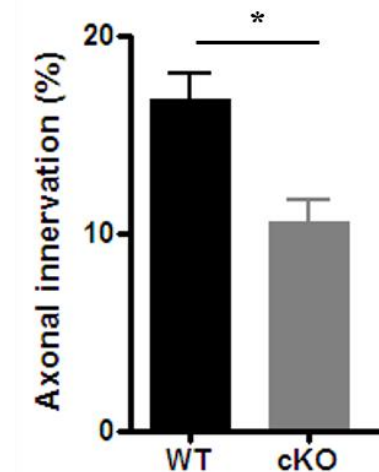
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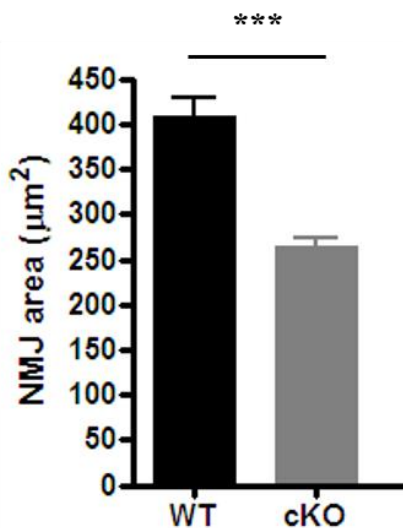
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Figure 3. Muscle-specific NRIP KO mice have smaller NMJ area and lower axonal innervation percentage in NMJ. (A) Whole-mounting immuno-fluorescent analysis using α-Bungarotoxin (α-BTX) and anti-neurofilament (NF) antibody to stain NMJ and its innervated axon, respectively. Acetylcholine receptors (AChR) are detected by α-BTX (red), neural axon are detected by anti-NF antibody (green), and muscle nuclei are detected by DAPI (blue). The results demonstrate that 12-16 weeks old cKO mice have abnormal morphology of NMJ and deficient axonal innervation compared with age-matched WT mice. Images was taken by confocal microscopy. There are five groups of representative images from both WT and cKO soleus whole-mounting tissue. Scale bar:

10 um. (B) Quantification of　NMJ area in WT and cKO mice. NMJ area is measured by ImageJ software. The results showed that NMJ area of cKO mice are significantly smaller than WT mice (WT: n=4, total counted NMJ: 95; cKO: n=4, total counted NMJ: 84). ***$p<0.001$, student's t test. (C) Quantification of　axonal innervation percentage in NMJ of WT and cKO mice. NMJ area and axon-terminal area innervating into NMJ were measured by ImageJ software. The proportion of axon-terminal area innervating into NMJ in NMJ area was calculated. The results demonstrated that the axonal innervation percentage in cKO NMJ is lower than in WT NMJ (WT: n=4, total counted NMJ: 95; cKO: n=4, total counted NMJ: 84). *$p<0.05$, student's t test.

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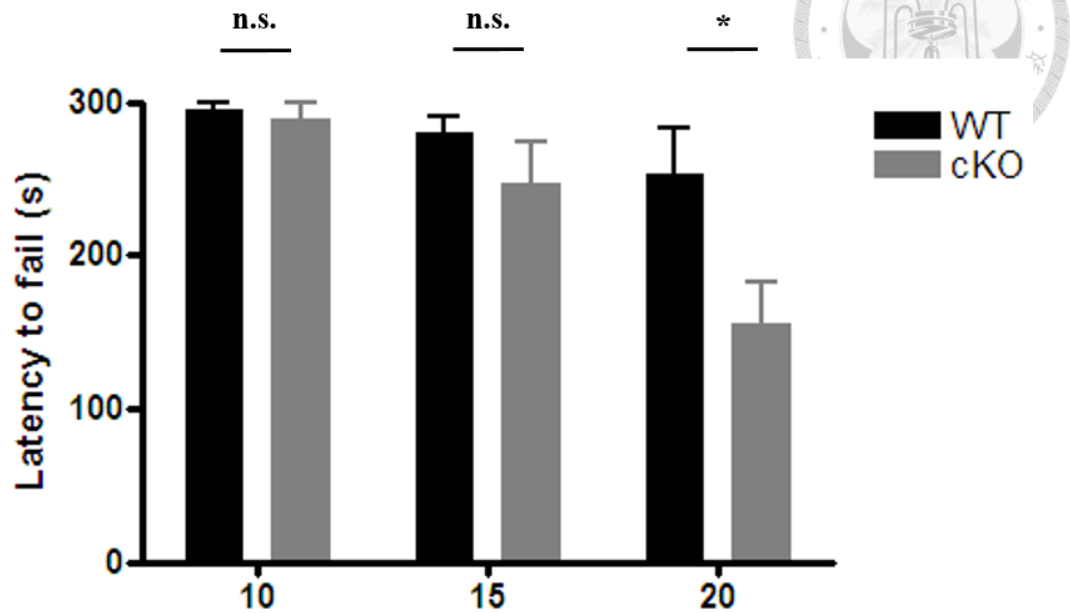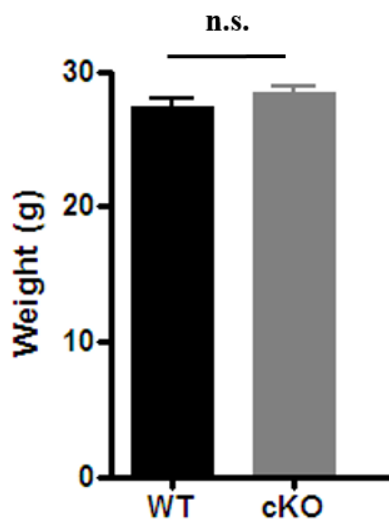
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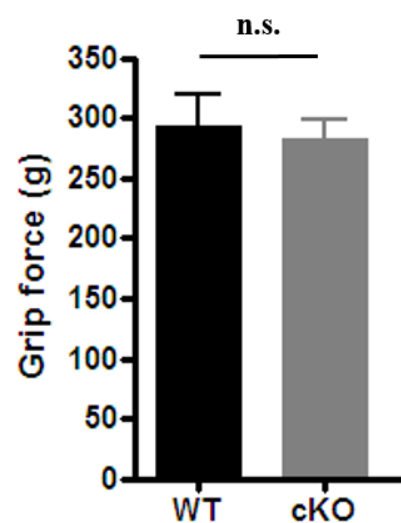
Figure 4. Behavior test in WT and cKO mice. (A) Rotard rod test. Both WT and cKO mice were placed on a rod rotating with increasing rate from 10 r.p.m., 15 r.p.m., to 20 r.p.m., and measured their riding time respectively at different rate. There is no significant difference between WT and cKO mice under the condition of 10 r.p.m. and 15 r.p.m. However, cKO mice has worse latency than WT mice under the condition of 20 r.p.m. *$p<0.05$, student's t test. (B) Body weight measurement. The statistic results revealed no

significant difference in body weight between 12-16 weeks old WT and cKO mice (WT: n=8, cKO: n=11). n.s., no significance, student's t test. (C) Grip force analysis. Muscle specific NRIP KO mice do not display deficient grip force in comparison to WT mice (WT: n=3, cKO: n=5). n.s., no significance, student's t test.
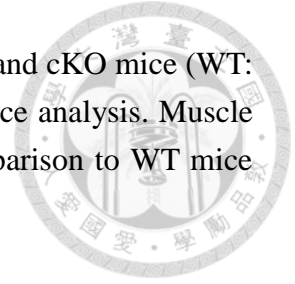
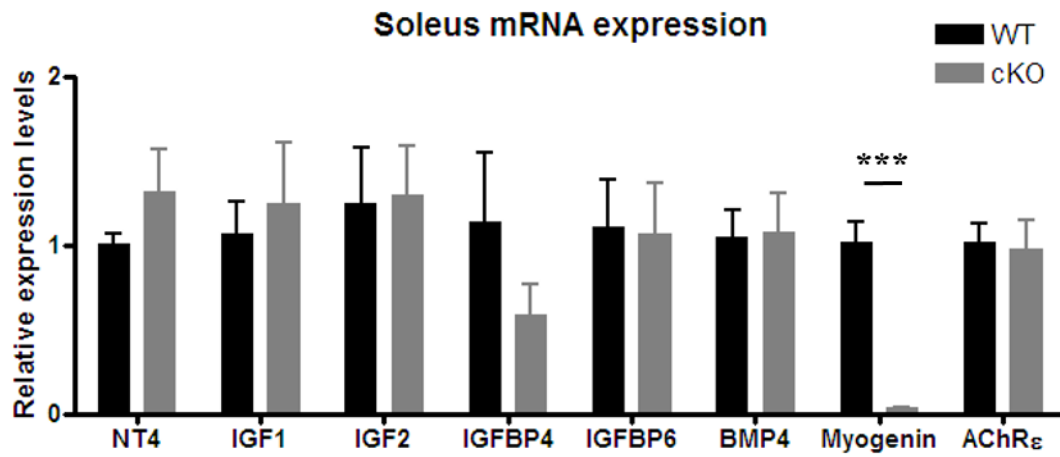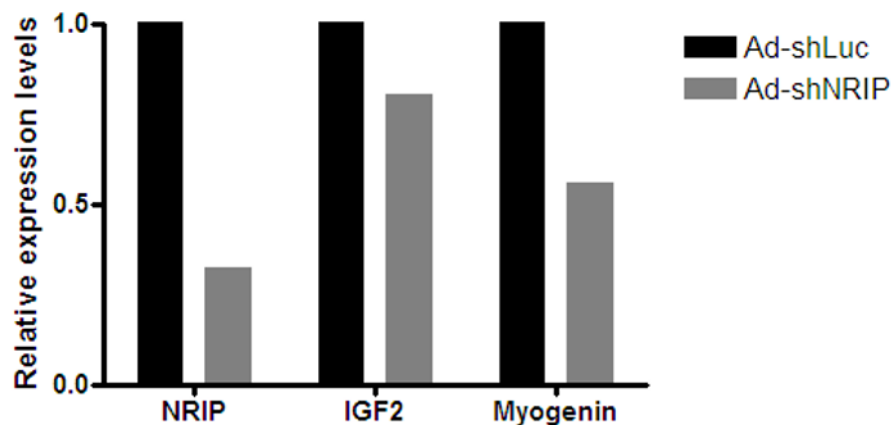**(A)**
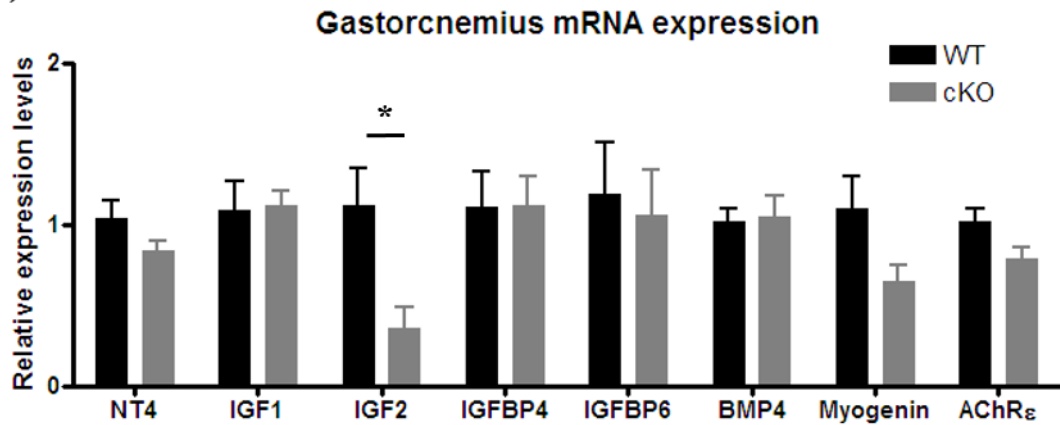
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Figure 5. Selection of muscle-derived candidate factors which may affect motor neuron survival. (A) Messenger RNA levels of various candidate genes in gastrocnemius tissue of WT and cKO mice. Gastrocnemius tissue mRNA were isolated and specific primers were used to measure mRNA levels of specific genes including NT4, IGF-1, IGF-2, IGFBP-4, IGFBP-6, BMP4, myogenin, and AChRε in a RT-qPCR reaction. The result showed that only IGF2 mRNA levels in gastrocnemius of cKO mice are significantly lower than that in WT mice (WT: n=6, cKO: n=7). There is no statistically difference in other genes between cKO and WT mice. *p<0.05, student's t test. (B) Messenger RNA levels of various candidate genes in solues tissue of WT and cKO mice. Soleus tissue mRNA were isolated and mRNA levels of the same genes in panel (A) were measured by RT-qPCR. The result showed that only myogenin mRNA levels in soleus of cKO mice are significantly lower than that in WT mice (WT: n=4, cKO: n=4). There is no statistically difference in other genes between cKO and WT mice. ***p<0.005, student's t test. (C) Measurement of IGF2 and myogenin mRNA levels in C2C12 cells. Differentiated C2C12 cells were infected with either Ad-shLuc or Ad-shNRIP, and then mRNA levels of IGF2 and myogenin were measured by RT-qPCR reaction. The result showed that NRIP knockdown in C2C12 cells decreases myogenin mRNA levels compared with control. In contrast, IGF2 mRNA levels does not display apparent change in C2C12 cells in which NRIP gene is knockdown in comparison with controls. Ad-shLuc: n=1, Ad-shNRIP: n=1.

Chapter 6 REFERENCES
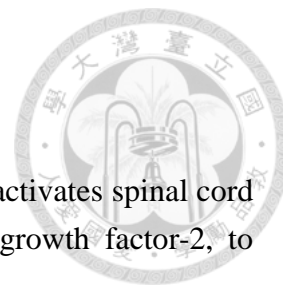
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