

Exploring functional roles of the MuSK-BMP pathway

By

Chengjie Xi

B.A. Biology, Grinnell College, 2022

Thesis

Submitted in partial fulfillment of the requirements for the Degree of Master of Science
in the Graduate Program of Biotechnology at Brown University

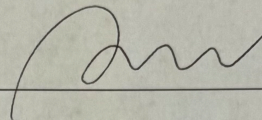
PROVIDENCE, RHODE ISLAND

March 2024

AUTHORIZATION TO LEND AND REPRODUCE THE THESIS

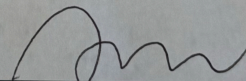
As the sole author of this thesis, I authorize Brown University to lend it to other institutions for the purpose of scholarly research.

Date 4/26/2024


Chengjie Xi, Author

I further authorize Brown University to reproduce this thesis by photocopying or other means, in total or in part, at the request of other institutions or individuals for the purpose of scholarly research.

Date 4/26/2024


Chengjie Xi, Author

This thesis by (Chengjie Xi) is accepted in its present form by the Graduate Program in Biotechnology as satisfying the thesis requirements for the degree of Master of Science

Date <u>4/22/24</u>	Signature: <u>[Signature]</u> Dr. Justin Fallon, Advisor
Date <u>4/26/24</u>	Signature: <u>[Signature]</u> Dr. Arturo Andrade, Reader
Date <u>4/22/2024</u>	Signature: <u>[Signature]</u> Dr. Jacquelyn Schell, Reader

Approved by the Graduate Council

Date _____ Signature: _____
Dr. Thomas Lewis, Dean of the Graduate School

Acknowledgments

I would like to express my deepest gratitude to my advisor, Dr. Justin Fallon, for accepting me as a master's student and for his invaluable mentorship, support, and encouragement throughout the last two years of my research at Brown University. His expertise, guidance, and insightful feedback have been instrumental in shaping this thesis. I would also like to acknowledge all the Fallon lab fellows for their support and collaboration, which have enriched my research experience. Special thanks to Beth McKechnie for her indispensable support and assistance with experimental design and reagent supplies throughout my research, to Dr. Laura Madigan, who taught me how to use cryostat and do IHC preps, and to Dr. Lauren Fish, who provided me with the opportunity to contribute to her NMJ projects, and always being around to share her knowledge and answer my questions about everything. In addition, I would like to thank Lexi-Amber Hassell and Kelsey Babcock of the Webb Lab for helping me with floating section staining and the generous sharing of antibodies.

Additionally, I am grateful to my readers, Dr. Arturo Andrade and Dr. Jacquelyn Schell, for being my committee members and taking the time to review my thesis.

To my friends, thank you for the laughter that lightened my days, for the encouragement whenever I needed it, and for always being proud of me, whoever I am. Lastly, I want to express my deepest gratitude to my family for all the unconditional love and support they have given to me. I would not have been able to come this far without them.

Table of Contents

<i>AUTHORIZATION TO LEND AND REPRODUCE THE THESIS</i>	<i>ii</i>
<i>Acknowledgments</i>	<i>iv</i>
<i>Table of Contents</i>	<i>v</i>
<i>Table of Figures</i>	<i>vi</i>
<i>Table of Tables.....</i>	<i>vii</i>
<i>Abstract.....</i>	<i>8</i>
<i>Chapter 1: Introduction</i>	<i>9</i>
MuSK and MuSK expression.....	9
The Delta Ig3 MuSK mouse as a tool to study the MuSK-BMP pathway.....	11
Roles of MuSK-BMP pathway.....	12
<i>Chapter 2: Methods</i>	<i>19</i>
Animals and Tissue harvesting	19
Immunohistochemistry, microscopy, and quantification.....	19
Anti-MuSK antibodies	21
Fresh Frozen Muscle/Brain Sections IHC	24
Brain Floating Sections IHC	25
<i>Chapter 3: Results</i>	<i>26</i>
MuSK-BMP pathway regulates the localization of voltage-gated sodium channels at NMJs	26
Increased fibrosis in Δ Ig3-MuSK soleus	28
Characterization of Anti-MuSK antibodies	29
Localization of MuSK protein in the brain	33
<i>Chapter 4: Discussion</i>	<i>40</i>
<i>References</i>	<i>43</i>

Table of Figures

Figure 1. MuSK signals through three distinct pathways.....	10
Figure 2. ΔIg3-MuSK mouse model	11
Figure 3. Depiction of the process of generating muscle fiber action potential (MFAP) and nerved-evoked muscle contraction at the neuromuscular junction	13
Figure 4. Composition of the subventricular zone (SVZ) in the lateral ventricle (LV).....	15
Figure 5. Composition of the subventricular zone (SVZ) in the lateral ventricle (LV).....	17
Figure 6. The intensity of voltage-gated sodium channels Nav1.4 reduced at ΔIg3-MuSK NMJs	27
Figure 7. Increased type I collagen levels in ΔIg3-MuSK soleus.	29
Figure 8. Summary of anti-MuSK antibodies.	30
Figure 9. Characterization of Human Anti-MuSK antibodies at NMJs of TA.....	32
Figure 10. Characterization of Rabbit Anti-MuSK antibodies at NMJs of TA.....	33
Figure 11. MuSK signals were detected at the subventricular zone (SVZ) of the lateral ventricle (LV) at 2 months of age	36
Figure 12. MuSK signals in putative NSCs at the subventricular zone (SVZ) of the lateral ventricle (LV) by anti-MuSK Ig3 antibody, 24C10 human at 8 months of age	37
Figure 13. MuSK signals were detected at the subgranular zone (SGZ) of the dentate gyrus (DG).....	39

Table of Tables

Table 1. Compiled list of primary antibodies and secondary antibodies for Anti-MuSK

IHC..... 22

Abstract of Exploring functional roles of the MuSK-BMP pathway, by Chengjie Xi, ScM, Brown University, May, 2024.

Muscle-specific tyrosine kinase (MuSK) is a transmembrane receptor tyrosine kinase first found at the post-synaptic domains of neuromuscular junctions (NMJs). It has long been known for its function in the formation and maintenance of NMJs through agrin-LRP4 signaling, which is dependent on the Ig1 domain of MuSK. Recently, the Fallon lab has shown that MuSK also shapes and augments BMP signaling through its binding to BMP and BMP co-receptors via the Ig3 domain. They termed this the MuSK-BMP pathway and generated a transgenic mouse model, 'ΔIg3-MuSK', which constitutively expresses MuSK lacking the Ig3 domain. With ΔIg3-MuSK as a tool, this work explores the functional roles of the MuSK-BMP pathway in various contexts, including the NMJ, where MuSK is enriched; the slow muscle, e.g., soleus, where MuSK is also known as expressed extrasynaptically; and the brain. Notably, MuSK expression is not well-characterized in the central nervous system. Here, we show that the density of voltage-gated sodium channel Nav1.4 reduced in ΔIg3-MuSK NMJs but not the sarcolemma, which explains the excitability defects previously found in ΔIg3-MuSK and indicates the role of the MuSK-BMP pathway in Nav1.4 localization at NMJs. Additionally, the level of type I collagen, a major component of extracellular matrix (ECM), was increased in the ΔIg3-MuSK soleus, suggesting the role of the MuSK-BMP pathway in fibrosis in the slow muscle. Finally, a panel of anti-MuSK antibodies for distinct domains and used for immunohistochemistry to localize MuSK protein in the brain. MuSK was detected in the subventricular zone (SVZ) of the lateral ventricle (LV) and the subgranular zone (SGZ) of the dentate gyrus, the two neurogenic niches in the brain. These data provide critical support for the role of the MuSK-BMP pathway in regulating adult neurogenesis.

Chapter 1: Introduction

MuSK and MuSK expression

Muscle-specific kinase (MuSK) is a transmembrane receptor first described as expressed at the postsynaptic membrane of all NMJs [1-3]. MuSK consists of an ectodomain with three immunoglobulin-like domains (Ig1, Ig2, and Ig3), one CRD/Frizzled-like domain, and a cytosolic domain dominated by its tyrosine kinase domain and a key regulatory juxtamembrane tyrosine [3]. MuSK has been best known for its role in the formation and maintenance of NMJs [1-3]. Agrin-LRP4/MuSK signaling and Dok7 downstream signaling, which is dependent on the binding of Ig1 domain of MuSK to LRP4 and tyrosine kinase activity, is critical for organizing of acetylcholine receptors (AChRs) in the postsynaptic domains (Fig. 1A) [3-9]. MuSK also participates in MuSK-Wnt signaling through the binding of Wnts to MuSK via the CRD/FZ domain to induce AChR clustering, which plays an important role in the development and maintenance of NMJ (Fig. 1B) [3,10,11]. Recently, the Fallon lab reported that MuSK is a BMP co-receptor that augments and shapes BMP signaling. The Ig3 domain is required for high affinity MuSK binding to BMPs and Type I BMP receptors, while tyrosine kinase activity is dispensable in this pathway (Fig. 1C) [3,12]. In addition to being expressed at all NMJs, MuSK is also expressed extrasynaptically in slow muscles (e.g., the soleus) but not in fast muscles like the tibialis anterior (TA) muscles [13].

In contrast to the muscle, the expression of MuSK in the brain is not well-characterized. In preliminary studies, the Fallon Lab has shown the expression of MuSK in isolated neural stem cells (NSCs) from mouse SVZ at both transcriptional and protein levels [18]. Recently, MuSK expression has also been shown to in NSCs isolated from the hippocampus (Choi, S., unpublished data). MuSK expression is also detected in hippocampal SGZ NSCs by single-cell

RNA sequencing [14]. There is also a study that reported the expression of MuSK in the brain by in situ hybridization [15]. However, there have been no studies looking at the localization of MuSK protein in the brain by immunohistochemistry using well-characterized and highly specific anti-MuSK antibodies.

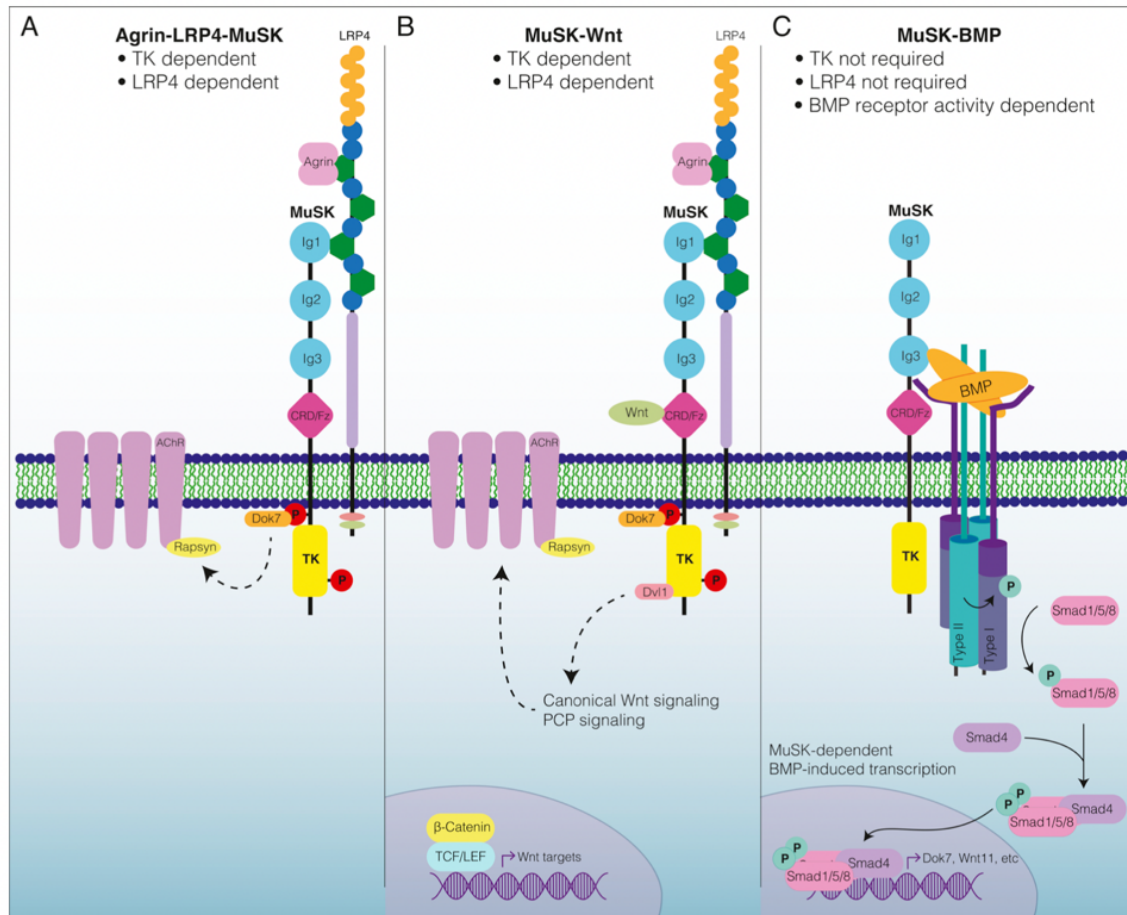


Figure 1. MuSK signals through three distinct pathways. (A) In the canonical Agrin-LRP4-MuSK pathway, neural agrin binds LRP4 via the Ig1 domain of MuSK to initiate Dok7 downstream signaling cascade resulting in anchoring of AChRs at the muscle membrane. (B) MuSK-Wnt pathway also affect AChR clustering dynamics. Wnts binds to MuSK via the CRD/Fz domain to activate both canonical Wnt and planar cell polarity cascades. (C) MuSK-BMP pathway. BMPs bind MuSK via the Ig3 domain. MuSK-BMP signaling induces Smad1/5/8

phosphorylation and induces expression of a set of MuSK-BMP dependent transcripts in myoblasts and myotubes. MuSK also binds Type I BMP receptors, but the domain mediating this interaction is currently unknown. The MuSK-BMP pathway does not require MuSK tyrosine kinase activity. Source: Figure and legend adapted from Fish and Fallon, 2020.

The Delta Ig3 MuSK mouse as a tool to study the MuSK-BMP pathway

To modulate MuSK-BMP signaling, the Fallon Lab generated a mouse model, “ Δ Ig3-MuSK”, that constitutively expresses MuSK lacking the Ig 3 domain, which is required for BMP binding [12]. The mouse was generated by using CRISPR-Cas9 to delete exons 6-7, which encode the MuSK Ig3 domain (Fig. 2) [16]. Homozygous Δ Ig3-MuSK mice are viable and fertile with normal life span. BMP4 signaling is reduced in cultured myogenic cells from Δ Ig3-MuSK mice. The mice showed similar grip strength and weight at 3 months of age [16]. This mouse model allows us to explore the functional roles of MuSK-BMP signaling in various contexts, including NMJs [17], slow muscles [16], and the brain [18].

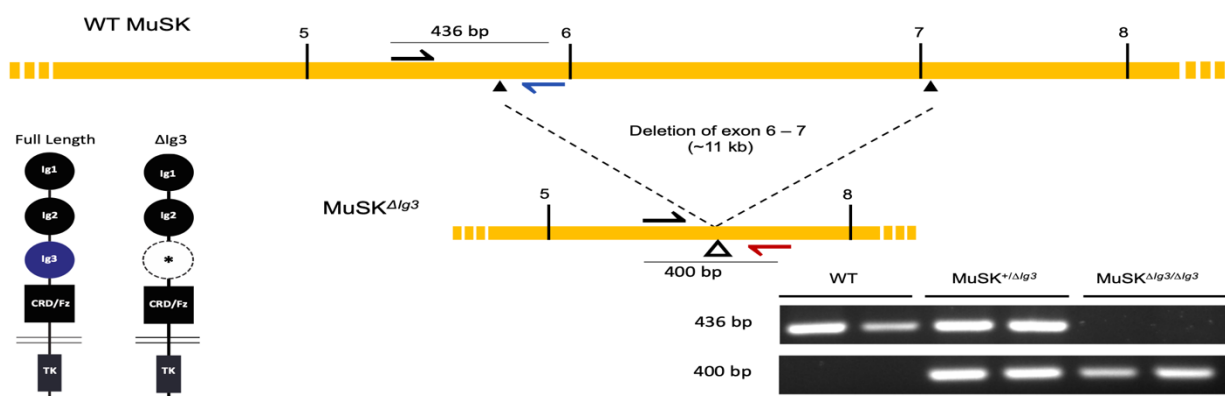


Figure 2. Δ Ig3-MuSK mouse model. Schematic depiction of generating Δ Ig3-MuSK mouse model by using CRISPR-Cas9 to delete exon 6-7 of MuSK, which encodes the Ig3 domain. PCR

amplification of WT and Δ Ig3-MuSK alleles in WT and Δ Ig3-MuSK heterozygous and homozygous mice using allele-specific primers. Source: Figure and legend adapted from Jaime et al., 2024.

Roles of MuSK-BMP pathway

NMJ, muscle excitability, and voltage-gated sodium channel

The NMJ is a specialized synapse that exchanges and stores information between a motor neuron and a muscle fiber. It plays an important role in the initiation of nerve-evoked muscle contraction by transducing a nerve-evoked action potential into a muscle fiber action potential (MFAP) [17]. Propagated action potentials along the nerve cause the release of acetylcholine (ACh) by the nerve terminal, allowing the acetylcholine receptors localize at the post-synaptic domain to depolarize and generate endplate potentials, which are in turn amplified by subadjacent voltage-gated Nav1.4 sodium channels in the postsynaptic domain to generate MFAPs and nerve-evoked muscle contractions (Fig. 3) [19-23]. With the Δ Ig3-MuSK mouse model, we were able to study the functional role of the MuSK-BMP pathway at NMJs. Δ Ig3-MuSK mice were found to have more fragmented post-synaptic NMJs in multiple muscle types compared with WT throughout the lifespan, but spontaneous and nerve-evoked ACh release as well as postsynaptic AChR clustering and currents in Δ Ig3-MuSK NMJs were all preserved [Fish et al., biorxiv, 17]. While Δ Ig3-MuSK demonstrated MFAP jitter and blocking measured by single-fiber electromyography (SFEMG), indicating deficits in the ability to induce muscle excitation by the nerve-evoked endplate potentials. The nerve-evoked muscle force was also reduced in Δ Ig3-MuSK NMJs [Fish et al., biorxiv, 17]. These findings all point to the direction of investigating the role of MuSK-BMP signaling in voltage-gated sodium channel localization since the generation of muscle contractions is highly dependent on the localization of muscle-

specific voltage-gated Na⁺ channels (Nav1.4) [24, 25]. As part of my thesis work, I collaborated with Lauren Fish, a former Ph.D. student in the Fallon Lab, to examine the role of the MuSK-BMP pathway in regulating voltage-gated sodium channels Nav1.4 at the NMJs.

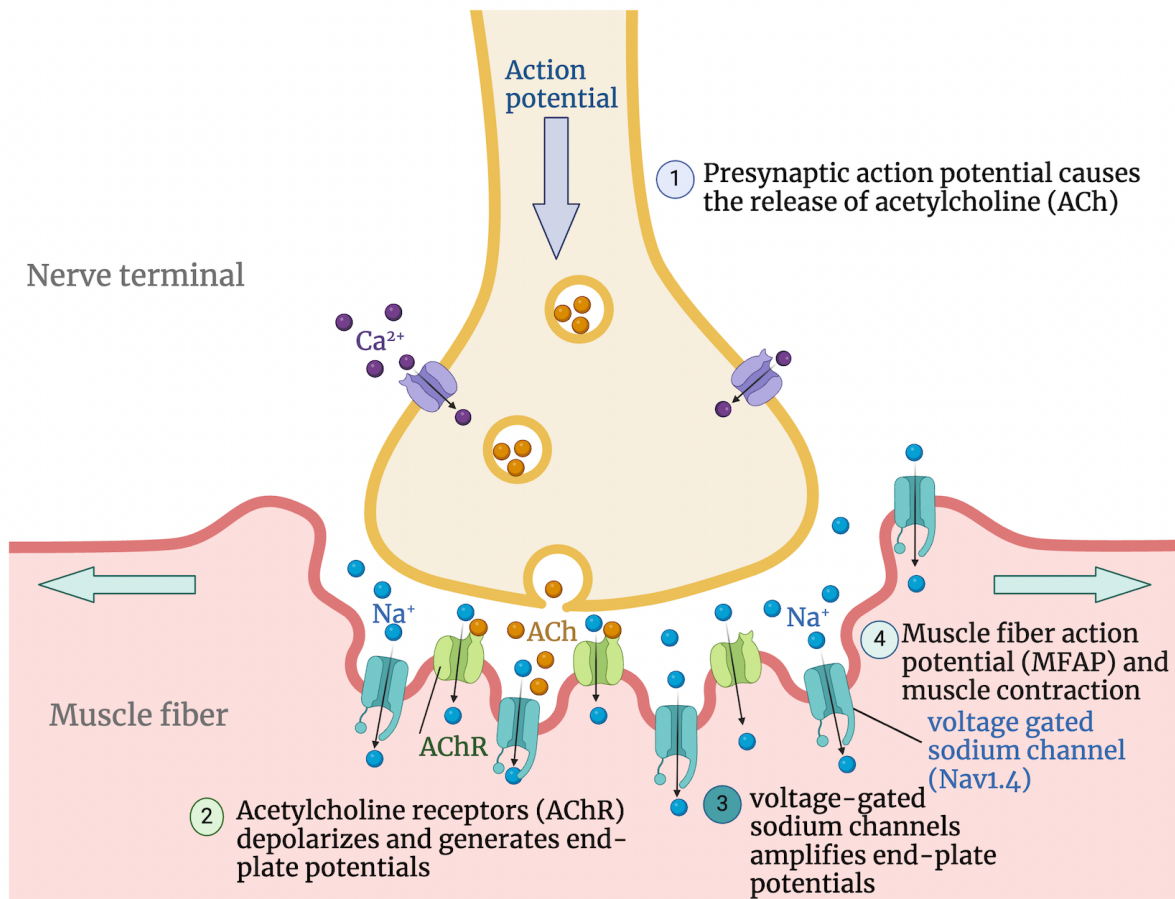


Figure 3. Depiction of the process of generating muscle fiber action potential (MFAP) and nerve-evoked muscle contraction at the neuromuscular junction. Presynaptic action potential propagates along the nerve and causes the release of ACh to the synaptic cleft. High densities of AChRs harboring at the post-synaptic membrane let Na⁺ in to depolarize and generate endplate potentials in response to the ACh release. Voltage-gated sodium channels (Nav1.4), also high

densities at post-synaptic membrane subadjacent to AChRs, amplify endplate potentials to generate MFAP and muscle contractions. Image created using Biorender.com.

Slow muscle, myofiber size, and fibrosis

Maintaining myofiber size is critical for muscle functions. BMP signaling is known as one of the positive regulators of myofiber size. Overexpression of BMP or the activity of BMP receptors promoted muscle growth, including increased muscle mass and fiber size [26]. The Fallon Lab recently reported that the MuSK-BMP pathway regulates the maintenance of myofiber size in slow muscle (e.g., soleus) selectively but not in fast muscle (e.g. TA) by promoting IGF1-Akt-mTOR signaling [16]. The Δ Ig3-MuSK mice showed the reduced size of myofiber in soleus but not in TA compared with WT at 3 months of age [16]. RNA-seq revealed that pathways involved in extracellular matrix (ECM) and collagen fibril organizations are upregulated in Δ Ig3-MuSK mice soleus [16]. This observation was supported by the WGA staining, which is a useful fibrosis marker that binds to various glycoproteins of the cell membrane, showing a striking thickening of extracellular matrix around myofibers [16, 27]. However, further verification was needed to identify specific molecular changes in Δ Ig3-MuSK mice soleus ECM. In this study, I examined the deposition of type I collagen in the Δ Ig3-MuSK soleus.

Adult neurogenesis and BMP signaling

Adult neurogenesis is the process of new neurons are generated throughout life in adult brains. There has been long-established evidence for several decades that adult neurogenesis occurs in rodents [28-34]. In rodents, there are two neurogenic niches. One is the subventricular zone (SVZ) located along the wall of the lateral ventricle (LV) [29]. Adult neural stem cells

reside in SVZ and rarely divide, but when they are activated, they give rise to transient amplifying progenitor cells and generate neuroblasts that will eventually migrate through the rostral migratory stream to the olfactory bulb [30] (Figure 4).

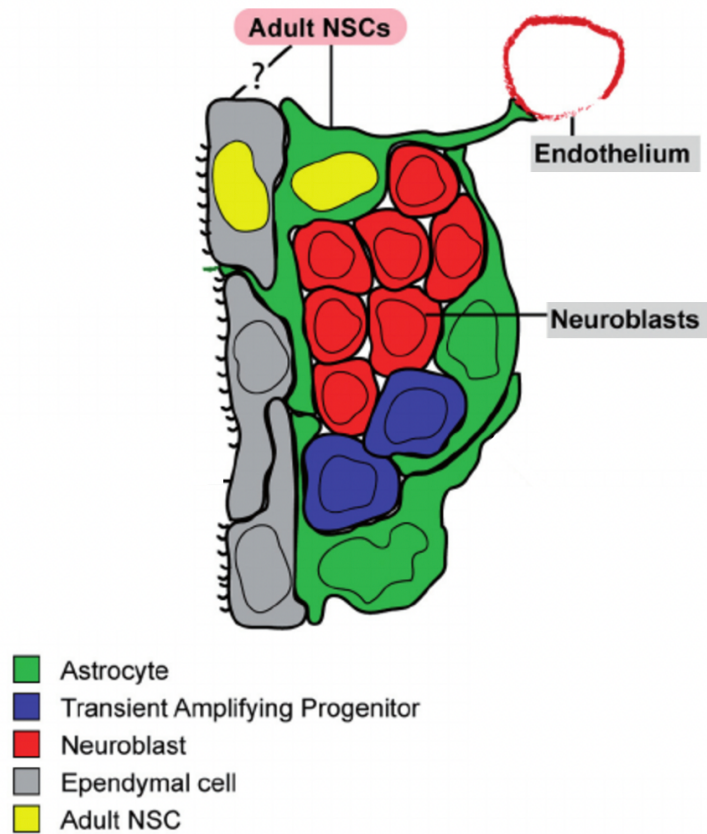


Figure 4. Composition of the subventricular zone (SVZ) in the lateral ventricle (LV).

Astrocytes (Green) are resident support cells of SVZ. Ependymal cells (Grey) are cells lining up the wall of LV. Adult NSCs are mostly quiescent. When they are activated, they will give rise to transient amplifying progenitors (Blue), which proliferate and generate neuroblasts (Red). Those neuroblasts will migrate through the rostral migratory stream to the olfactory bulb. Figure and legend adapted from Basak and Taylor, 2008.

The other neurogenic niche is the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus [28, 42]. More recently, several lines of evidence from multiple labs have

detected adult neurogenesis also occurs in the hippocampus of the adult human brain using various methods, including immunohistochemistry, carbon-14 dating, and high-resolution magnetic resonance imaging [35-41]. Adult hippocampal neurogenesis has critical functions, including learning and memory, cognition, and emotional regulation [37,40,41]. Neural stem cells in the adult hippocampus are largely quiescent, but they can be activated, proliferate, and differentiate to immature neurons, which are highly plastic and have critical function (Fig. 5) [42].

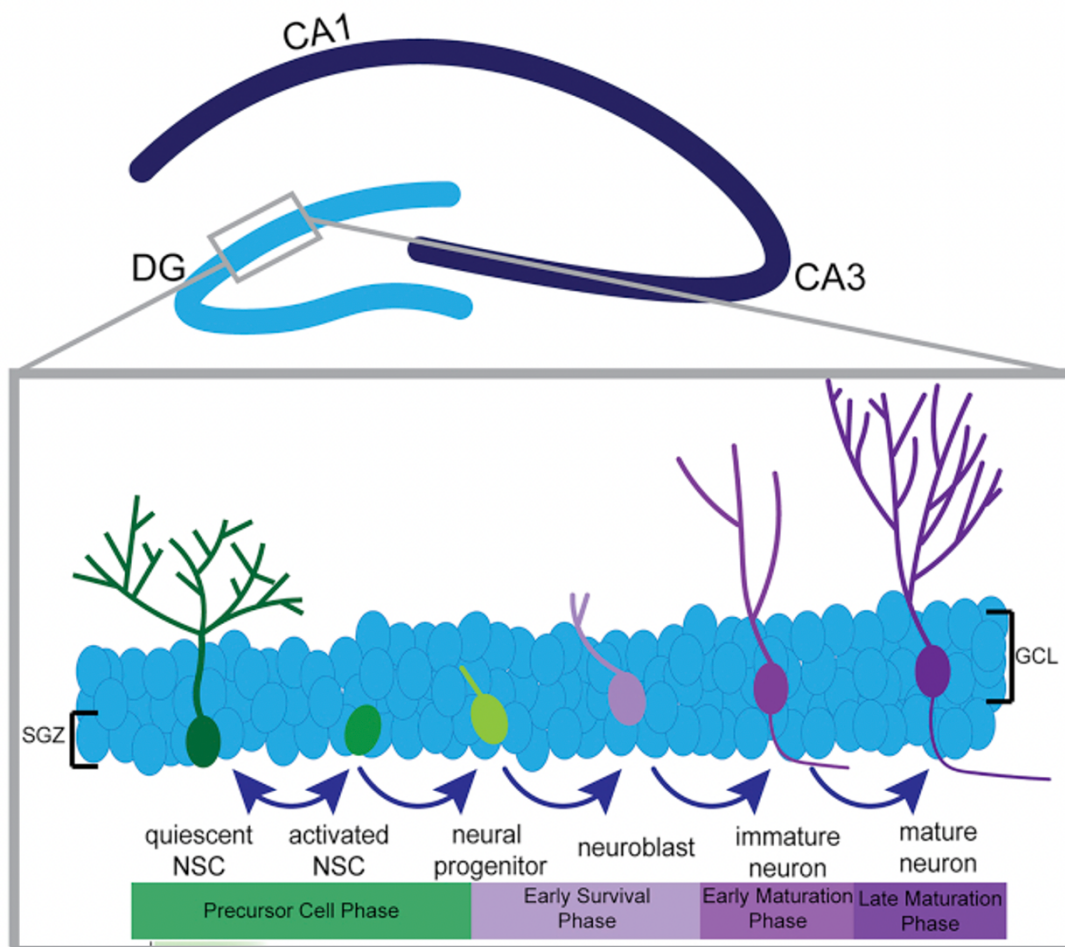


Figure 5. Composition of the subventricular zone (SVZ) in the lateral ventricle (LV).

Astrocytes (Green) are resident support cells of SVZ. Quiescent NSCs that become activated proliferate to daughter cells that either differentiate into neural progenitor cells (or glia) or return to quiescence for replenishment of the NSC pool. Survived neural progenitor cells beyond the early survival stage integrate into the mature circuitry. Source: Figure and legend adapted from Babcock et al., 2021.

There are multiple factors that could regulate AHN. For example, physical exercise, such as voluntary running, has been shown to promote neurogenesis in adult mice [31]. Living in an enriched environment with increased sensory, cognitive, and motor stimulation has also been demonstrated to lead to increase AHN in mice [31]. In addition, chronic treatment with Selective Serotonin Reuptake Inhibitors (SSRIs), a class of antidepressants, and exposure to young blood through parabiosis or plasma transfer are also known as positive regulators of AHN [43, 44]. In contrast, AHN declines in multiple neurological conditions, including aging, neurodegenerative disease, and psychiatric disease, e.g., major depression, brain injury, and drug exposure, and contributes to cognitive dysfunction [38,42,45,46].

BMP signaling is one of the best-understood signaling pathways that negatively regulate AHN. BMPs, bone morphogenic proteins, are negative signals in the neurogenic niche that inhibit both the activation of quiescent NSCs and the integration of immature neurons [47-51] (See also Fig. 5). BMP signaling is also found to increase in Alzheimer's disease, major depression, inflammatory conditions, and aging [51-53]. Thus, inhibiting BMP signaling could have therapeutic implications for those neurological conditions associated with impaired neurogenesis, such as depression, Alzheimer's disease, and other cognitive disorders.

However, BMPs, their receptors, and their antagonists (e.g., noggin) are widely expressed and have diverse roles throughout the body, making it a challenge to target BMP for modulating neurogenesis selectively. Our approach is to take advantage of the MuSK-BMP pathway, which has a highly restricted expression pattern. The Fallon lab, collaborated with the Webb lab, has shown that Δ Ig3-MuSK mice exhibit increased AHN for about >2.5 fold assessed by EdU/DCX+ study, a widely used assay for assessing AHN in rodents, and improved hippocampal-dependent spatial memory by Novel Object Location (NOL) task [18], indicating the critical role of MuSK-BMP pathway in regulating AHN. Although there have been studies reporting the expression of MuSK in the hippocampus by single-cell RNA seq [14], and our preliminary study showed the expression of MuSK in isolated NSCs from SVZ. However, the specific cell types that express MuSK, especially in the neurogenic niche. In addition, is there any other brain region that expresses MuSK remains unknown, causing the need for localization of MuSK in the brain. In this work, I characterized a panel of anti-MuSK antibodies and used them to localize the expression of MuSK in the brain with immunohistochemistry (IHC).

Chapter 2: Methods

Animals and Tissue harvesting

All animal protocols were conducted under regulations set by and with the approval of the Brown Institutional Animal Care and Use Committee. Δ Ig3-MuSK mice were generated as described in Jaime et al., 2022 [16]. WT mice were C57BL/6 mice from The Jackson Laboratory (JAX). Animals for muscle harvest were euthanized via CO₂ inhalation followed by cervical dislocation. Muscles were harvested, flash-frozen in liquid-nitrogen-frozen isopentane and stored at -80° C. Animals harvested for fresh frozen brain were anesthetized with Avertin (25 μ l/g) and trans-cardiac perfused with PBS only. Fresh frozen brains were flash-frozen in liquid-nitrogen-frozen isopentane, embedded in optimum cutting temperature (OCT) media, and stored at -80° C. Animals harvested for floating sections of brain were anesthetized with Avertin (25 μ l/g) and trans-cardiac perfused with PBS followed by ice-cold 4% paraformaldehyde (PFA), which was freshly made in PBS on the day of perfusion, post-fixed in 4% PFA overnight at 4° C, dehydrated in 30% sucrose/water 4° C, and embedded in optimum cutting temperature (OCT) media and stored at -80° C. Fresh frozen brains were perfused with PBS only, flash-frozen in liquid-nitrogen-frozen isopentane, embedded in optimum cutting temperature (OCT) media and stored at -80° C.

Immunohistochemistry, microscopy, and quantification

Anti-Nav1.4 and Anti-AChR

WT and Δ Ig3-MuSK TA and gastrocnemius muscles were harvested at 3 months and 6 months of age. Fresh frozen muscles were cross-sectioned on a cryostat (Leica) at 10 μ m and transferred onto microscope slides. Cryosections were fixed in 4% paraformaldehyde (PFA) in PBS for 5 min. After washing 3 $\times$ 5 min with PBS, sections were permeabilized with 0.5%

Triton X-100 in PBS for 10 min and subsequently incubated in blocking buffer (5% normal goat serum, 0.2% BSA in PBS) for 1 hr. Sections were incubated with the primary anti-Nav1.4 antibody (1:1000, NeuroMab/Antibodies Inc N255/38) overnight at 4 °C. After washing 3×5 min with PBS containing 0.1% Triton X-100, sections were detected with secondary antibody (Alexa fluor 594 Goat anti-Mouse IgG2a, Invitrogen, A-21135), and counterstained with Alexa Fluor 488-conjugated α -bungarotoxin (Thermo Fisher, B13422) diluted in blocking buffer at 1:1000 for 1 hr at room temperature. After washing 3×5 min with PBS containing 0.1% Triton X-100, sections were mounted with Vectashield antifade mounting medium with DAPI. Images were acquired with a Nikon E800 fluorescence microscope at 60X objective in a single session, using the same exposure for all slides for quantitative immunofluorescence analysis. In FIJI/ImageJ (NIH), I manually traced the entire neuromuscular junction or similarly sized sarcolemmal areas (extrajunctional area) based on the Nav1.4 signal and measured the mean intensity of Nav1.4 and AChR separately at the same traced region of interest for the same exposure. For background subtraction, images were acquired from the center of the same myofiber bearing the synapse in each channel. The intensity of Nav1.4 and AChR signals at neuromuscular junctions or extrajunctional area, minus the background, of WT and Δ Ig3-MuSK TA muscles were compared by measuring their mean intensity. Ten neuromuscular junctions were analyzed each for WT and Δ Ig3-MuSK muscles. (n=6) Statistical testing (unpaired Student's T-test) was conducted in Prism 9.

Anti-Collagen 1 Alpha 1

WT and Δ Ig3-MuSK soleus were harvested at 3 months of age. Fresh frozen 10 μ m cross-sections were incubated with the primary antibody rabbit anti-Collagen 1 alpha 1 (Novus biologicals NB600-1052) as described above. Images were acquired with a Nikon E800

fluorescence microscope at 60X objective in a single session, using the same exposure for all slides for quantitative immunofluorescence analysis. For quantification, I manually traced the periphery of myofiber and measured the mean fluorescence intensity of Collagen 1 Alpha 1 in FIJI/ImageJ (NIH). Background intensity was obtained from the center of the same myofiber bearing the periphery. Normalized mean intensities are mean intensities of Collagen 1 alpha 1 minus the background. Three segments of different myofibers were analyzed for five sections of WT and Δ Ig3-MuSK soleus muscles (n = 6). Statistical testing (unpaired Student's T-test) was conducted in Prism 9.

Anti-MuSK antibodies

We used human antibodies specific for each of the three immunoglobulin-like domains (Ig1, Ig2, and Ig3) of MuSK (Table 1., See also Fig.8). We obtained an anti-MuSK Ig1 antibody from Maartje G. Huijbers at Leiden University [54]. This antibody is purified human IgG4 antibodies from MuSK-MG patients. MuSK-MG, MuSK-associated Myasthenia Gravis, is an autoimmune disease characterized by patients having circulating antibodies against the Ig1 and Ig2 domains of MuSK [54-56], which interfere with the MuSK-Agrin-LRP4 pathway and cause NMJ impairments [3-11]. These antibodies undergo affinity maturation in the patient's body, so they have a very high affinity to MuSK. We got two anti-MuSK Ig2 antibodies, 189-1 and 189-28, from Kevin C. O'Connor at Yale University. These anti-MuSK Ig2 antibodies are human monoclonal antibodies cloned from MuSK-MG patient B cells and produced recombinantly [55]. We also have two anti-MuSK Ig3 antibodies, 24C10 mouse and 24C10 human, from Aimee S. Payne at Columbia University. One is mouse IgG obtained from hybridoma produced by immunizing the mice with the human MuSK Ig3 domain, and another one is a human anti-MuSK that was produced recombinantly from mouse IgG [56]. Bolden Therapeutics also provided 5

rabbit polyclonal anti-peptide or anti-fusion protein antibodies against the different cytosolic domains of MuSK (Table 1., See also Fig.8).

Table 1. Compiled list of primary antibodies and secondary antibodies for Anti-MuSK IHC.

Antibody name	Antibody Types	Antigen recognized	Source	Reference	Comments
11-3F6	Human IgG4 Fraction from MuSK MG Patients	MuSK Ig1	Gift M. Huijbers	54	Only work with fresh frozen muscle/brain sections, can't withstand with PFA-perfusion fixation.
189-1	Human recombinant monoclonal antibody	MuSK Ig2	Gift K. O'Connor	55	Only work with fresh frozen muscle/brain sections, can't withstand with PFA-perfusion fixation.
189-28	Human recombinant monoclonal antibody	MuSK Ig2	Gift K. O'Connor	55	Not very specific, unspecific signals seen in TA staining (data not shown).
24C10 mouse	Mouse IgG	MuSK Ig3	Gift A.Payne	56	Work with PFA-perfusion fixed brain floating sections. M.O.M kit required.
24C10 human	Human IgG4 produced recombinantly	MuSK Ig3	Gift A. Payne	56	Only work with fresh frozen

	from mouse IgG				muscle/brain sections, can't withstand with PFA-perfusion fixation.
Rb 2a, 2b	Rabbit polyclonal	MuSK juxta membrane cytosolic (a.a. 539-556)	Bolden Therapeutics		Not very specific, unspecific signals seen in TA staining (data not shown).
Rb 12	Rabbit polyclonal	MuSK TK domain (a.a. 660-673)	Bolden Therapeutics		Specificity confirmed at NMJs.
Rb 4a, 4b	Rabbit polyclonal	MuSK C-term of cytosolic domain (a.a. 857-870)	Bolden Therapeutics		Not very specific, unspecific signals seen in TA staining (data not shown).
Rb 5a, 5b	Rabbit polyclonal	MuSK entire cytosolic domain (a.a. 518-869)	Bolden Therapeutics		5b: Specificity confirmed at NMJs. 5a: Not very specific
Normal human IgG	IgG from human serum		Sigma Aldrich, I4506		
Normal rabbit IgG	IgG from rabbit serum		Sigma Aldrich, NI01		
Mouse anti-wrapper	Mouse monoclonal	Wrapper of Drosophila	DSHB 10D3		
Rb Anti-GFAP	Rabbit polyclonal	Glial Fibrillary Acidic Protein (GFAP)	Sigma Aldrich, AB5603		
Rb Anti-SOX2	Rabbit polyclonal	Transcription factor SOX-2	Sigma Aldrich, AB5804		

Alexa Fluor 488-conjugated α -bungarotoxin	Bungarus multicinctus venom	AChR	Thermo Fisher, B13422		
Rhodamine conjugated α -bungarotoxin	Bungarus multicinctus venom	AChR	Invitrogen T1175		
Alexa Fluor 488 Donkey Anti-Human	Donkey polyclonal	whole molecule human IgG	Jackson ImmunoResearch Laboratories, 709-545-149		
Alexa Fluor 555 Goat Anti-Human	Goat polyclonal	whole molecule human IgG	Thermo Fisher, A-21433		Cross-react with rabbit and mouse primaries
Alexa Fluor 555 Goat Anti-Rabbit	Goat polyclonal	whole molecule mouse IgG	Thermo Fisher, A-21428		
Alexa Fluor 555 Goat Anti-mouse	Goat polyclonal	whole molecule mouse IgG	Thermo Fisher, A32727		Cross-react with human primaries

Fresh Frozen Muscle/Brain Sections IHC

Fresh frozen 10 μ m cross-sections of WT and Δ Ig3-MuSK TA and 20 μ m fresh frozen brain coronal sections of WT and Δ Ig3-MuSK at various ages were used for specificity characterization. Muscle staining was conducted as described in Nav1.4 staining, except incubating with different anti-MuSK antibodies (1:1000 dilution) and counterstained with Alexa Fluor 488-conjugated α -bungarotoxin (1:1000 dilution) or Rhodamine (1:1000 dilution) depending on different secondary antibodies used (Table 1). Fresh frozen brain sections (20 μ m) staining was conducted in the same way as muscle staining while counterstaining with different markers for neural stem cells, including rabbit anti-GFAP (1:1000 dilution) and rabbit anti-

SOX2 (1:250 dilution). Images were acquired with a Nikon E800 fluorescence microscope at various objectives.

Brain Floating Sections IHC

Two-month-old PFA-perfusion-fixed mouse brains were cross-sectioned on a cryostat (Leica) at 40 μ m and stored in cryoprotectant [40% Phosphate Buffer (0.05M, pH 7.4), 30% ethylene glycol, and 30% glycerol] at -20 °C. Floating sections were washed 3 $\times$ 5 min with PBS and incubated in with M.O.M (Mouse on Mouse) Immunodetection Kit (Vector Laboratories, BMK-2202) for 1 hour before primary incubation. Sections were then incubated with 24C10 mouse (undiluted) overnight at 4°C, with gentle shaking. After washing 3X 15 min in 1X PBS + 0.01% Triton X-100, sections were incubated with Alexa flour 555 Goat-anti-mouse, 1:1000 dilution in M.O.M diluent, for 2 hours at room temperature. After washing 3X 15 min in 1X PBS + 0.01% Triton X-100, sections were then mounted on microscope slides with Vectashield antifade mounting medium with DAPI. Images were acquired with a Nikon Ti-2E fluorescence microscope at 20X objective.

Chapter 3: Results

MuSK-BMP pathway regulates the localization of voltage-gated sodium channels at NMJs

As described above, the generation of nerve-evoked muscle excitability is highly dependent on the localization of voltage-gated sodium channels Nav1.4 at the post-synaptic domains of NMJs [24, 25]. We hypothesized that the impairments in muscle excitability of Δ Ig3-MuSK were caused by the reduced level of Nav1.4. In Lauren Fish's wholemount staining of sternomastoid of WT and Δ Ig3-MuSK, Nav1.4 levels were most concentrated at the edges of the areas positive for AChR within the endplate (Fig. 6A) [17]. To quantify the Nav1.4 level, I used immunohistochemistry together with epi-fluorescence microscopy to compare the intensities of Nav1.4 at NMJs of WT and Δ Ig3-MuSK mice TA at 6 months of age. The result showed that the fluorescence intensity of Nav1.4 was reduced in Δ Ig3-MuSK compared to WT at the NMJ by ~27% ($p < 0.0001$) (Fig. 6C) with extrajunctional Nav1.4 levels preserved (Fig. 6D), indicating the role of MuSK-BMP pathway in regulating the localization of Nav1.4 at NMJs.

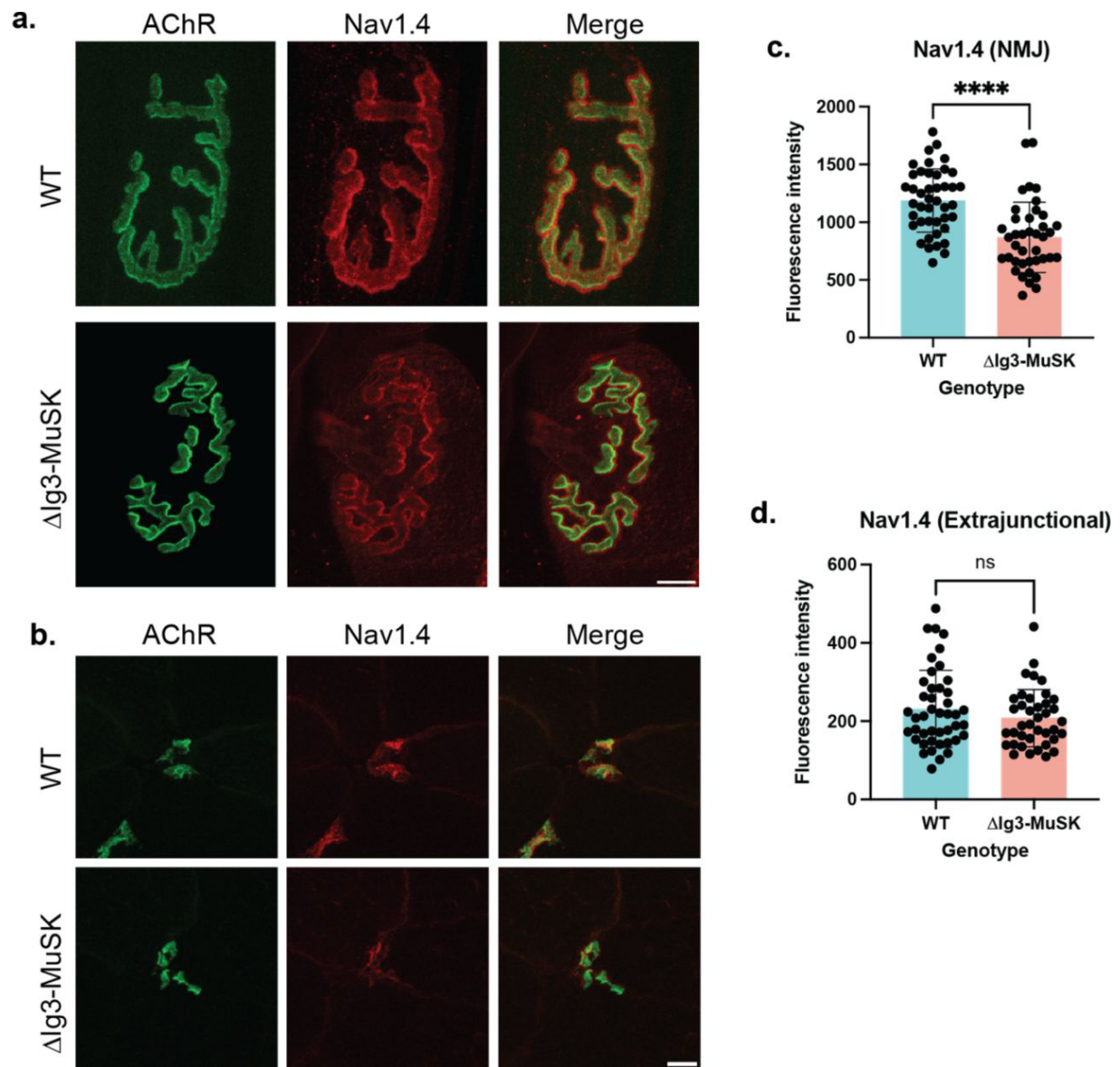


Figure 6. The intensity of voltage-gated sodium channels Nav1.4 reduced at Δ Ig3-MuSK NMJs. A. Immunostaining of WT and Δ Ig3-MuSK sternomastoid whole-mounts at 6 months of age by Lauren A. Fish. AChR in green and Nav1.4 in red. B. Immunostaining of WT and Δ Ig3-MuSK sternomastoid TA cross sections at 6 months of age. AChRs in green and Nav1.4 in red. C. Quantification of Nav1.4 fluorescence intensity showed a statistically significant reduction at Δ Ig3-MuSK NMJs ($p < 0.0001$, unpaired T-test, $n = 43$ NMJs from 4 WT mice, 40 NMJs from 4 Δ Ig3-MuSK mice). d. Extrajunctional Nav1.4 fluorescence intensity was preserved

in Δ Ig3-MuSK compared to WT ($p=0.22$, unpaired T-test, $n=44$ regions of interest from 4 WT mice, 39 regions of interest from 4 Δ Ig3-MuSK mice). Scale bar, 10 μ m. Figure and legend adapted from Fish et al., biorxiv, 2023.

Increased fibrosis in Δ Ig3-MuSK soleus

As described in the introduction that Diego Jaime's RNA-seq data of that ECM and collagen fibril organization GO pathways upregulated in Δ Ig3-MuSK mice soleus and the result of increasing WGA staining in Δ Ig3-MuSK mice soleus [16]. However, the major components that increased in the soleus were unknown. So, I investigated the distribution and level type I collagen, a major component of ECM, in WT and Δ Ig3-MuSK mice soleus at 3 months of age. The level of interstitial type I collagen increased for 49% in Δ Ig3-MuSK soleus compared to WT (Fig. 7A,B), confirming that there is fibrosis in the Δ Ig3-MuSK soleus.

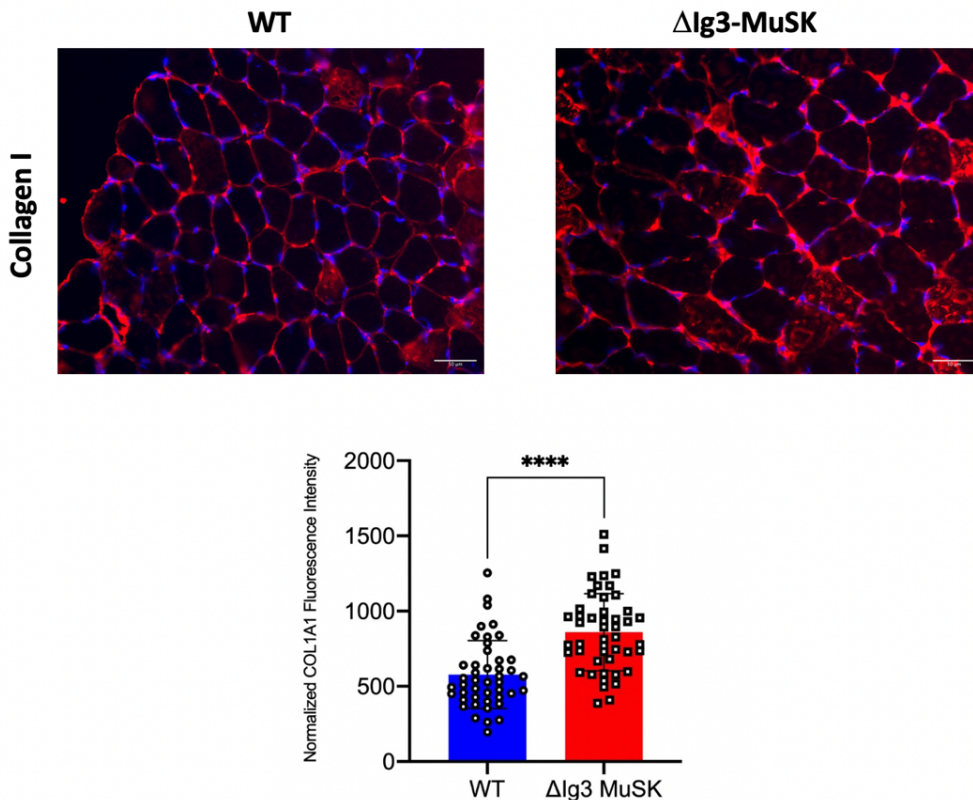


Figure 7. Increased type I collagen levels in Δ Ig3-MuSK soleus. *A. Immunostaining of WT and Δ Ig3-MuSK soleus cross sections at 3 months of age. Type I Collagen (red) and DAPI (blue). Scale bar, 50 μ m. B. Quantification of Type I collagen fluorescence intensities. The level of interstitial type I collagen was increased by 49% in the Δ Ig3-MuSK soleus (577.6 ± 33.7 , $n=47$ and 860.6 ± 37.3 , $n=45$ in WT and Δ Ig3-MuSK respectively, **** $p < 0.0001$, unpaired t -test; $n=6$ muscles per genotype; 5-6 sections/muscle). Figure and legend adapted from Jaime et al., 2024.*

Characterization of Anti-MuSK antibodies

To characterize the specificities of all the anti-MuSK antibodies I obtained from various sources (Fig. 8), I started with anti-MuSK immunostaining from in cross-sections of TA muscles, where we know there is abundant expression of MuSK at NMJs. The specificities of the anti-MuSK Ig1 antibody, 11-3F6, and anti-MuSK Ig2 antibody, 189-1, were confirmed by co-localization of the MuSK signal with the AChR signal at NMJs and negative signals in immunological control staining (Fig. 9A). The anti-MuSK Ig3 antibody, 24C10 human, was used in the TA of both WT and Δ Ig3-MuSK, which is the negative genetic control used here. The specificity of anti-MuSK Ig3 antibody, 24C10 human, was further confirmed with co-localization of MuSK signal with AChR signal at WT NMJs while no MuSK signal was observed at NMJs of Δ Ig3-MuSK NMJs (Fig. 9B). I have also identified two rabbit polyclonal anti-MuSK antibodies with good specificities at NMJs, 12 and 5b, judging by co-localization with AChR signals (Fig. 10).

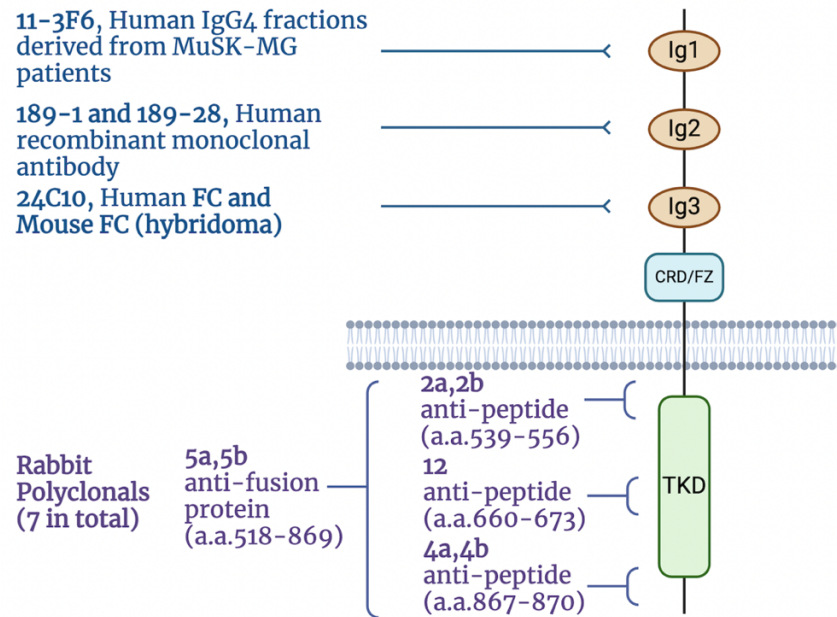


Figure 8. Summary of anti-MuSK antibodies. A panel of human antibodies against different Ig domains of MuSK from three generous labs (Maartje G. Huijbers, Leiden University; Kevin C. O'Connor, Yale University; Aimee S. Payne, Columbia University). Seven rabbit polyclonal antibodies against the cytosolic domain of MuSK. Image created using Biorender.com.

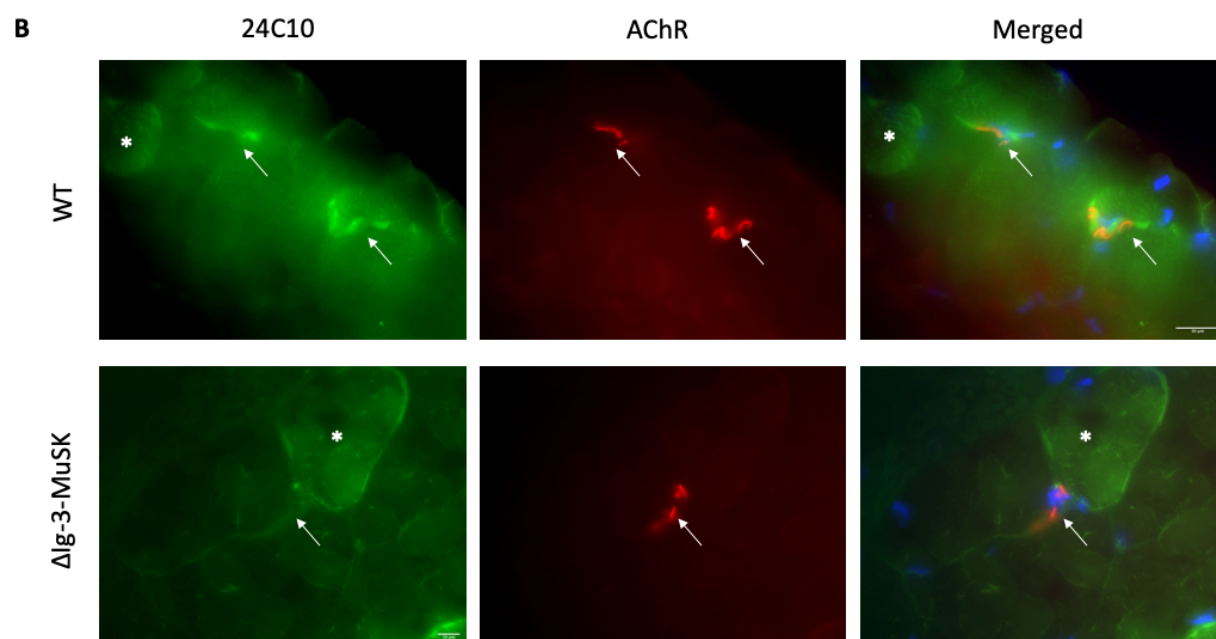
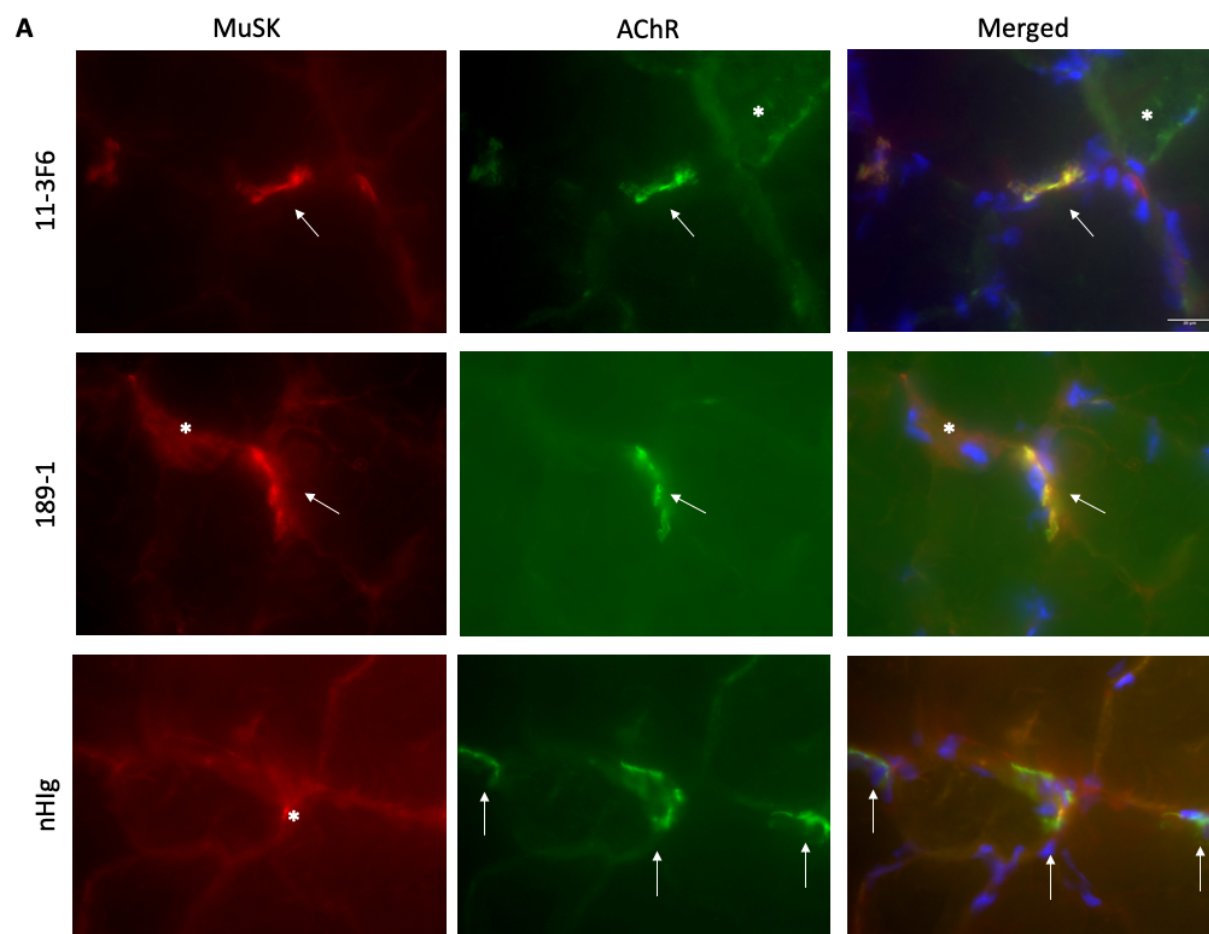


Figure 9. Characterization of Human Anti-MuSK antibodies at NMJs of TA. *A. Anti-MuSK immunostaining of WT TA muscle cross-sections. Specificities of anti-MuSK Ig1 antibody, 11-3F6, and anti-MuSK Ig2 antibody, 189-1, confirmed by co-localization with AChR signals at NMJs (pointed by white arrows). Immunological control, normal human IgG serum (nHIg) staining showed negative signal in red channel, further confirming the specificities. MuSK (Red), AChR(green), DAPI (blue). B. Anti-MuSK immunostaining of WT and Δ Ig3-MuSK (negative control) TA muscle cross-sections with anti-MuSK Ig3 antibody, 24C10. The specificity of 24C10 was confirmed by co-localization with AChR signals at NMJs (pointed by white arrows) and no MuSK at NMJs of Δ Ig3-MuSK. MuSK (green), AChR(red), DAPI(blue). Scale bar, 20 μ m, *non-specific staining, white arrows pointing NMJs (based on AChR signals).*

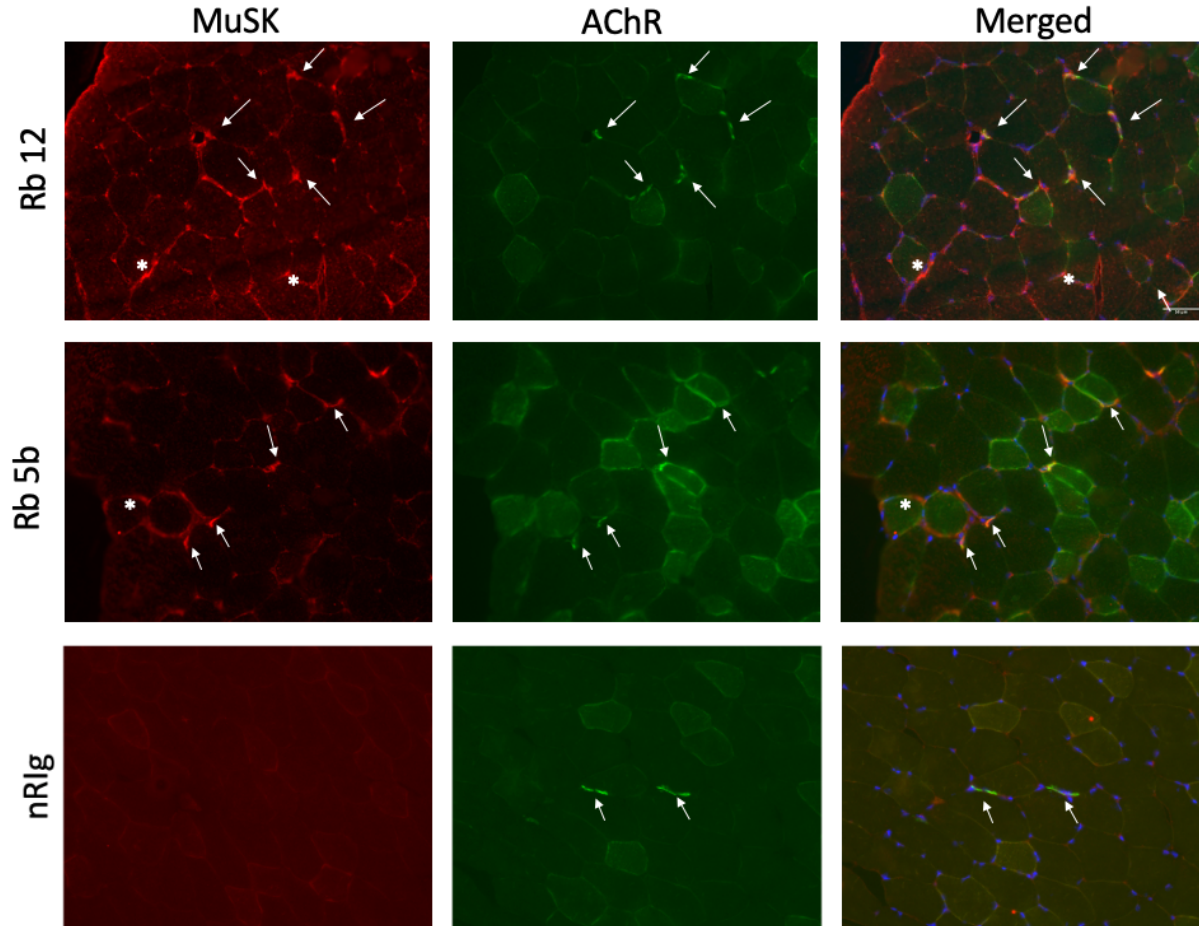
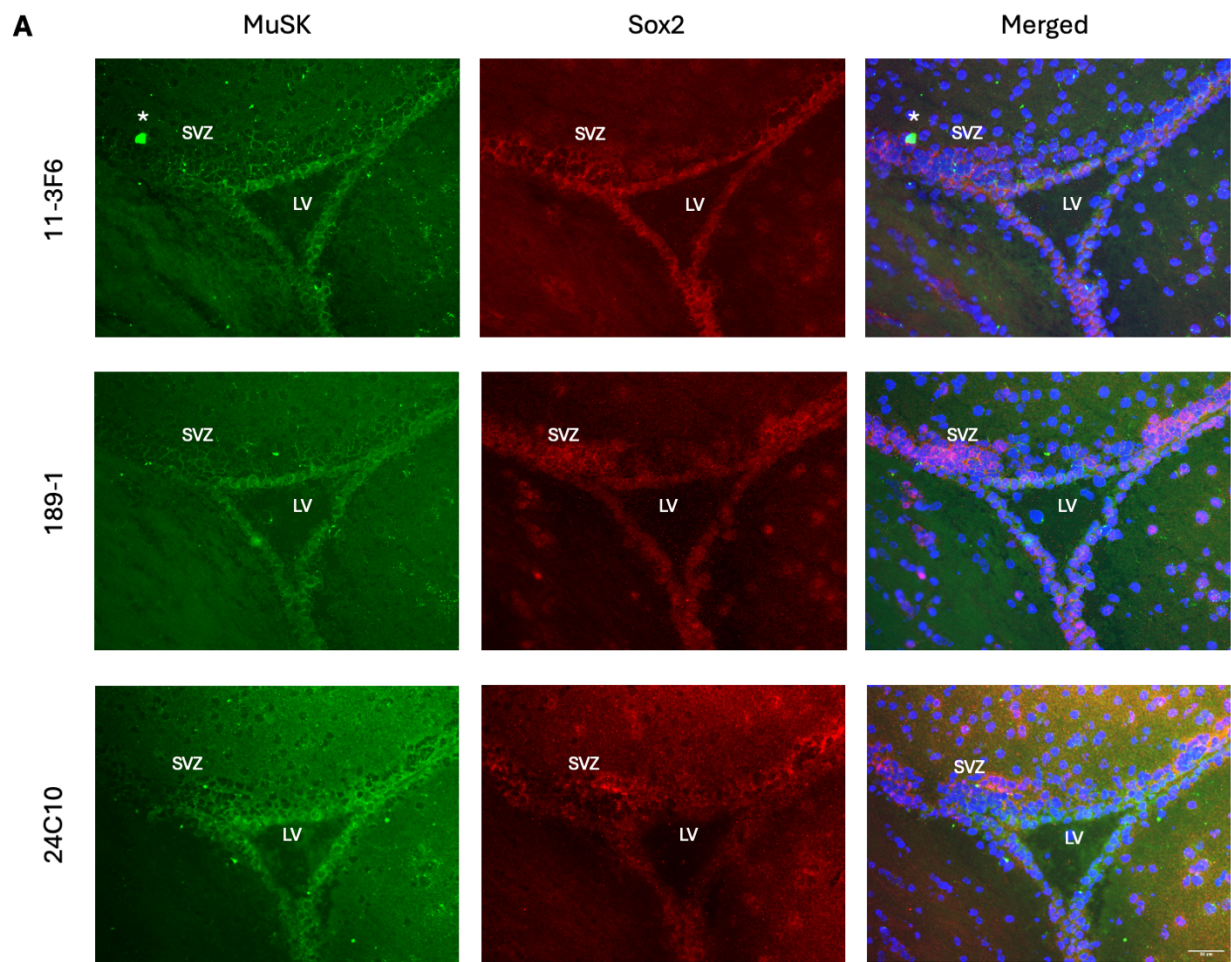


Figure 10. Characterization of Rabbit Anti-MuSK antibodies at NMJs of TA. Immunostaining of WT TA cross-sections with rabbit polyclonal anti-MuSK antibodies, Rb 5b, Rb 12, and normal rabbit IgG serum (negative control). Specificities confirmed by co-localization with AChR signals at NMJs (pointed by white arrows). No MuSK signals were observed at the NMJs of normal rabbit IgG staining. MuSK (Red), AChR (green), DAPI (blue). Scale bar, 50 μ m, *non-specific staining, white arrows pointing NMJs (based on AChR signals).

Localization of MuSK protein in the brain

With the panel of anti-MuSK antibodies that have characterized good specificities, I next used immunohistochemistry to investigate the expression of MuSK in the mouse brain, where

little is known about MuSK localization. I first characterized the rabbit polyclonal anti-MuSK antibodies (5b and 12). They showed differences from each other, suggesting that they were cross-reacting with non-MuSK proteins (not shown). Such cross-reaction was supported by Blast searches, which showed that the immunogen sequences for these antibodies were highly homologous or identical to several non-MuSK proteins. Therefore, for the work here, we focused on the human anti-MuSK antibodies (11-3F6, 189-1, and 24C10 human) and the mouse antibody (24C10 mouse). MuSK signals were detected in the subventricular zone (SVZ) of the lateral ventricle, which is one of the neurogenic niches (Fig. 10. A, B; See also Fig. 4). In 2-month-old mice, I observed MuSK signals in both ependymal cells lining up the LV and cells in SVZ of two-months-old WT, where neural stem cells (NSCs) are enriched as judged by NSC markers, SOX2, and GFAP (Fig. 10A,B). In contrast, at 8 months old, MuSK signals were only seen in putative neural stem cells at SVZ but not in ependymal cells of LV (Fig. 11A). I also confirmed the specificities with negative signals in both immunological and genetic control (Fig. 10C and Fig. 11B), which further confirmed the results. MuSK signal was also detected in the subgranular zone (SGZ) of the dentate gyrus, which is the other neurogenic niche, with the mouse anti-MuSK Ig3 antibody (24C10 mouse) (Fig. 12A), and no signals were seen with the immunological control (Fig. 12B).



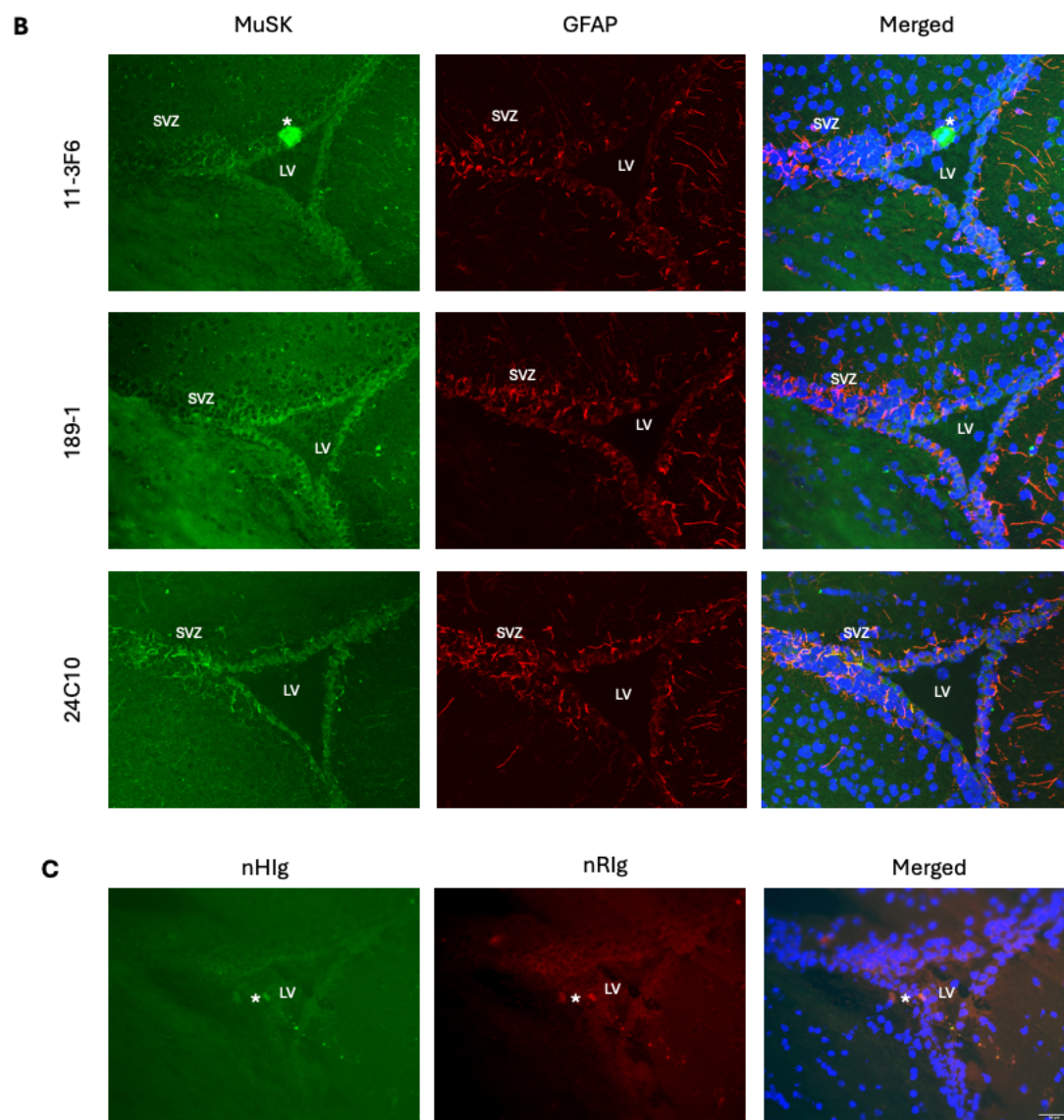


Figure 11. MuSK signals were detected at the subventricular zone (SVZ) of the lateral ventricle (LV) at 2 months of age. *A. Immunostaining of WT fresh frozen mouse brain sections (2 month-old) with human anti-MuSK antibodies: 11-3F6, anti-MuSK Ig1; 189-1, anti-MuSK Ig2, 24C10, anti-MuSK Ig3, and neural stem cell marker, Rb anti-SOX2. MuSK signals were detected both at the subventricular zone (SVZ), where neural stem cells are enriched, and ependymal cells lining up the lateral ventricle (LV). MuSK (Green), SOX2 (Red), DAPI (blue).*

Scale bar, 50 μ m, *non-specific staining. B. Immunostaining of WT fresh frozen mouse brain sections (2 month-old) with human anti-MuSK antibodies and Rb anti-GFAP, glial cell marker expressed by both NSCs and glial cells. MuSK signals detected in both SVZ and ependymal cells of LV. MuSK (Green), GFAP (Red), DAPI (blue). Scale bar, 50 μ m, *non-specific staining. C. Immunological control staining of normal human IgG serum (nHIg) and normal rabbit IgG serum (nRIg). No similar staining patterns of anti-MuSK were seen in the immunological control. nHIg (Green), nRIg (Red), DAPI (blue). Scale bar, 50 μ m, *non-specific staining.

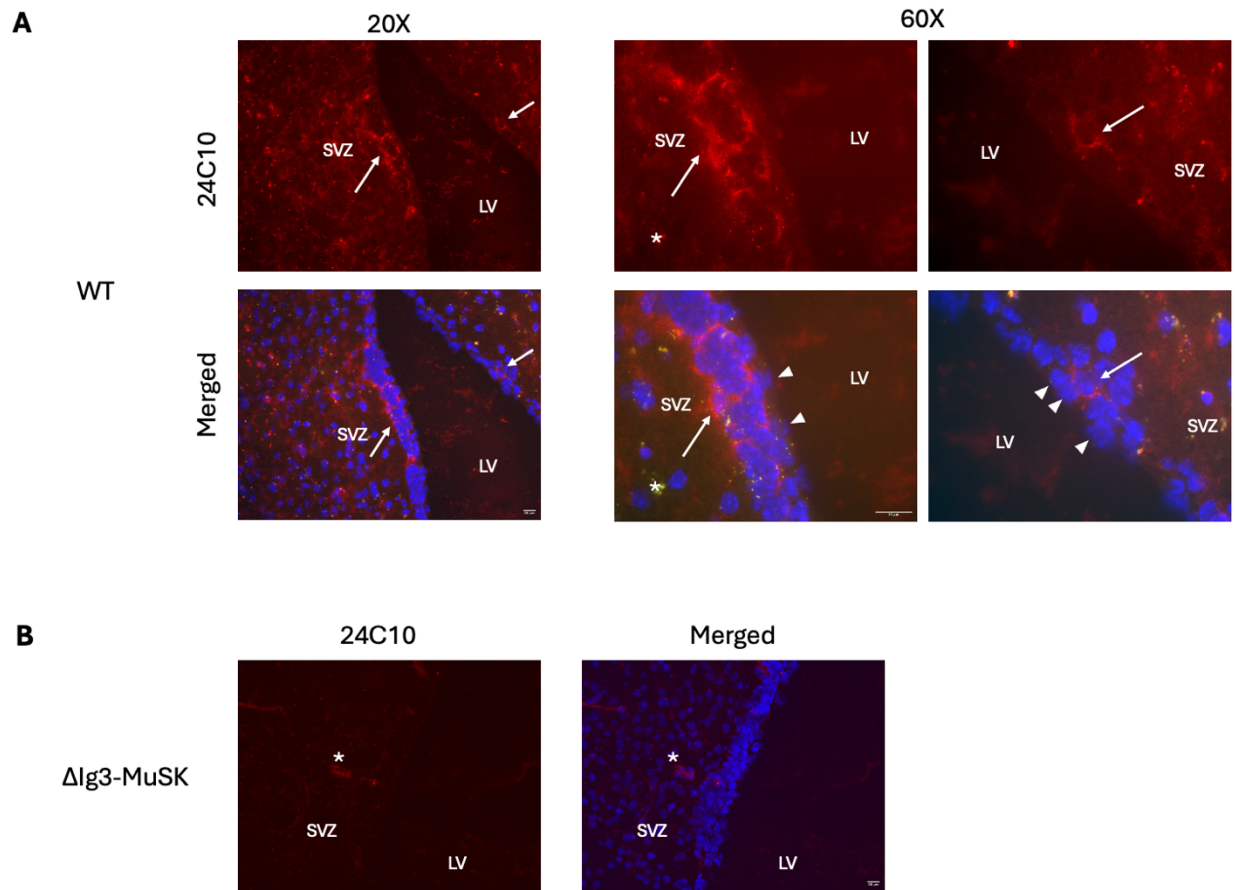


Figure 12. MuSK signals in putative NSCs at the subventricular zone (SVZ) of the lateral ventricle (LV) by anti-MuSK Ig3 antibody, 24C10 human at 8 months of age. A.

Immunostaining of WT (8-month-old) fresh frozen mouse brain sections with human anti-MuSK

*Ig3 antibody, 24C10. MuSK signals were detected in putative NSCs at the SVZ, while no signal was detected in the ependymal cells of LV. MuSK (Red), DAPI (blue). Scale bar, 20 μ m, *non-specific staining, white arrows indicate MuSK-positive putative NSCs, arrowheads indicate putative ependymal cells. B. Immunostaining of genetic control Δ Ig3-MuSK (9-month-old) fresh frozen mouse brain sections with human anti-MuSK Ig3 antibody, 24C10. No similar staining patterns of anti-MuSK were seen in the genetic control. MuSK (Red), DAPI (blue). Scale bar, 20 μ m, *non-specific staining.*

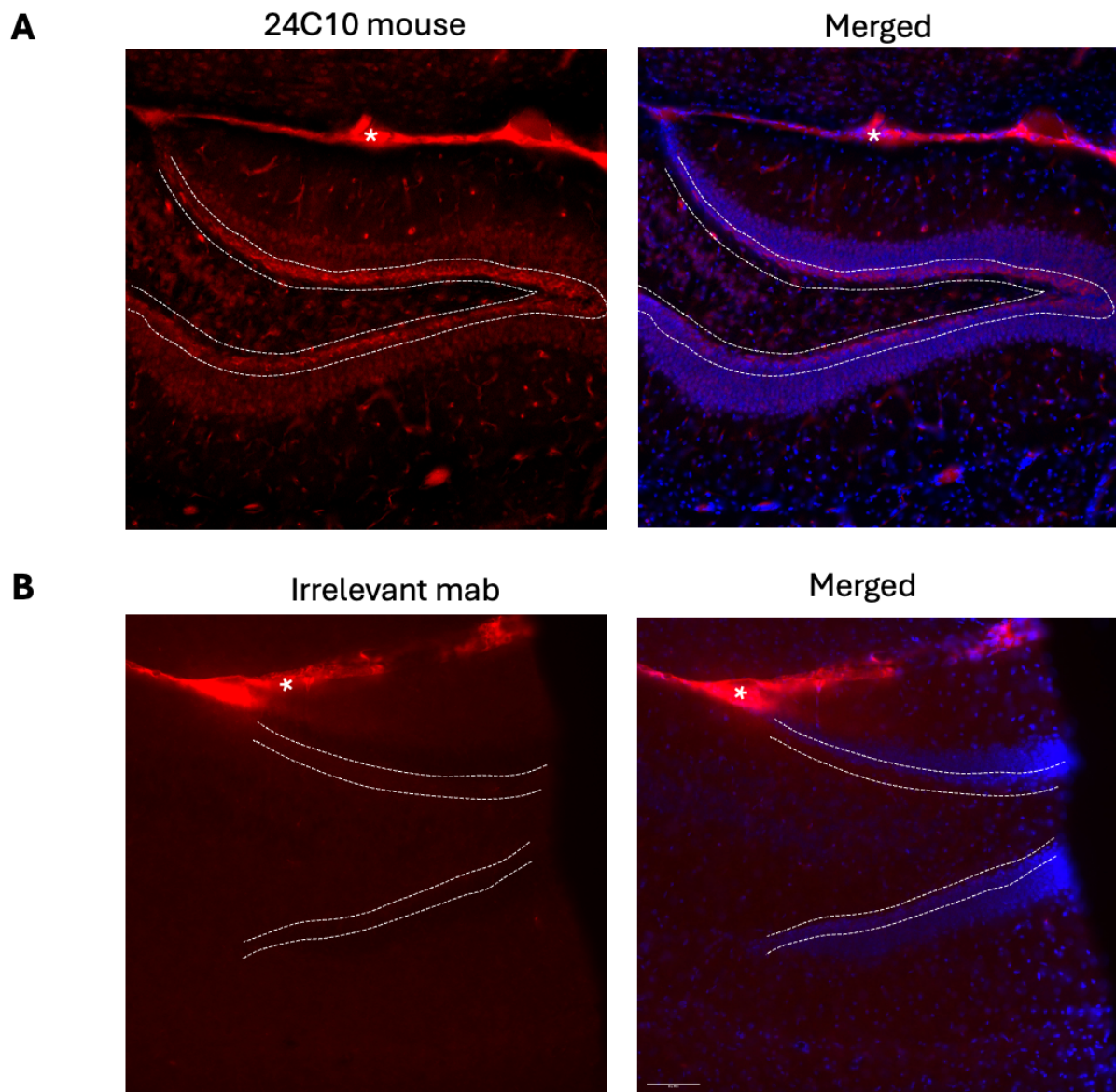


Figure 13. MuSK signals were detected at the subgranular zone (SGZ) of the dentate gyrus (DG). *A. Immunostaining of WT (2-month-old) mouse brain floating sections (40 μ m) with anti-MuSK Ig3 mouse antibody, 24C10. MuSK signals were observed in the SGZ of DG. B. Immunological control staining with an irrelevant mouse anti-wrapper (drosophila protein) antibody showed no signals at SGZ, further confirming the MuSK signals seen with 24C10 mouse. MuSK (Red), DAPI (blue). Scale bar, 50 μ m, dashed lines indicating SGZ *non-specific staining.*

Chapter 4: Discussion

In my thesis work, I investigated the functional roles of the MuSK-BMP pathway in various contexts, including NMJs, slow muscles, e.g., the soleus, and the neurogenic niches in the brain, primarily with immunohistochemistry.

Utilizing immunostaining and epi-fluorescence microscopy, I developed a method to quantify the level of different proteins with muscle cross-sections. With the quantification method, I was able to show that the level of voltage-gated sodium channel Nav1.4 reduced significantly at the NMJs of Δ Ig3-MuSK compared to WT, with extrajunctional Nav1.4 levels preserved (Fig. 6) [17]. This result helps explain the excitability defect of Δ Ig3-MuSK NMJs, which was revealed by increased jitter and blocking in response to nerve-evoked MFAPs while the cholinergic functions were normal at the Δ Ig3-MuSK NMJs [17], indicating the role of MuSK-BMP pathway selectively regulates localization of voltage-gated sodium channel Nav1.4 at the NMJs.

With the same quantification method, I have also shown the level of interstitial type I collagen increased by 49% in Δ Ig3-MuSK soleus compared to WT (Fig. 7), confirming the gene set enrichment analysis (GSEA) result using GO terms of RNA sequencing data that pathways regulating collagen fibril organization are upregulated in Δ Ig3-MuSK soleus [16].

In contrast to the long-established knowledge of MuSK in muscles, localization of MuSK expression in the brain is required for us to understand the functional role of the MuSK-BMP pathway in neurogenic niches. I have characterized a panel of anti-MuSK antibodies (11-3F6, 189-1, 24C10, Rb12, and Rb5b) with good specificities at NMJs where we know MuSK expression is enriched (Fig. 8 and Fig. 9). Results were further confirmed with immunological and genetic controls (Fig. 8 and Fig. 9). This characterization allows me to move on to use IHC

to investigate the localization of MuSK expression in the brain with those anti-MuSK antibodies I have characterized.

My results show that MuSK is expressed in the SVZ of the LV, which is one of the neurogenic niches in the murine brain. At 2 months old, MuSK signals were observed in both the ependymal cells lining up the wall of the LV and putative neural stem cells in the SVZ (Fig. 10). This result is supported by single-cell RNA seq data that MuSK is expressed in ependymal cell clusters of mouse SVZ [57]. It also verifies the preliminary result that isolated NSCs from SVZ express MuSK at both protein and transcript levels [18]. In contrast, at 8 months old, MuSK signals were only detected in the putative neural stem cells but not ependymal cells in the SVZ. It is noteworthy that SVZ neurogenesis does decline from 4 months of age [58], indicating that the loss of MuSK expression in the ependymal cells might be related to the decline of SVZ neurogenesis and the role MuSK-BMP pathway in SVZ neurogenesis.

I also showed that MuSK is expressed in the SGZ of the DG, the other neurogenic niche of the mouse brain (Fig. 12). This observation is consistent with previous single-cell RNA seq data that MuSK expression was detected in the hippocampus [14]. This result further supports Fallon Lab's work on manipulating the MuSK-BMP pathway to promote AHN [18] by adding evidence that MuSK is present in the neurogenic niche in the hippocampus. The limitation here is my results of SGZ staining only included mouse brains at 2 months old. It will be interesting to know if there is any age difference in the MuSK expression in the SGZ similar to what we observed in the SVZ.

For future directions, further experiments of IHC at different ages are needed to understand more about the change in MuSK expression in the SVZ in aging. Higher-quality microscopy imaging, e.g. Confocal, is required to show better co-localization of MuSK signals in

different cell types in the SVZ with different cell markers, such as ependymal cell markers and neuroblast markers. For the 24C10 mouse anti-MuSK Ig3 antibody, additional staining with our genetic control, Δ Ig3-MuSK, is needed to verify the MuSK signals detected in the SGZ. It is also important to have further anti-MuSK IHC with different cell markers to determine which cell type expresses MuSK protein in the SGZ at various ages. These all will help us better understand the role of the MuSK-BMP pathway in adult hippocampal neurogenesis.

References

1. DeChiara, T.M., et al., *The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo*. Cell, 1996. 85: p. 501-512.
2. Burden, S.J., Yumoto, N., and Zhang, W., *The role of MuSK in synapse formation and neuromuscular disease*, Cold Spring Harb. Perspect. Biol., 2013. **5**: a009167.
3. Fish, L.A. and Fallon, J.R., *Multiple MuSK signaling pathways and the aging neuromuscular junction*. Neurosci Lett, 2020. 731:135014.
4. Zhang, W., et al., *Agrin binds to the N-terminal region of Lrp4 protein and stimulates association between Lrp4 and the first immunoglobulin-like domain in muscle-specific kinase (MuSK)*. J. Biol. Chem., 2011. **286**: p. 40624-40630.
5. Zong, Y., et al., *Structural basis of agrin – LRP4 – MuSK signaling*. Genes Dev., 2012. **4**: p. 247-258.
6. Stiegler, A.L., et al., *Crystal structure of the agrin-responsive immunoglobulin-like domains 1 and 2 of the receptor tyrosine kinase MuSK*. J. Mol. Biol., 2006. **364**: p. 424-433.
7. Huijbers, M.G., et al., *MuSK IgG4 autoantibodies cause myasthenia gravis by inhibiting binding between MuSK and Lrp4*. Proc Natl Acad Sci U S A., 2013. 110: 20783-20788.
8. Lin, W., et al., *Distinct roles of nerve and muscle in postsynaptic differentiation of the neuromuscular synapse*. Nature, 2001. **410**: p. 1057-1064.
9. Yang, X., et al., *Patterning of muscle acetylcholine receptor gene expression in the absence of motor innervation*. Neuron, 2001. 30: p. 399-410.
10. Strohlic, L., et al., *Wnt4 participates in the formation of vertebrate neuromuscular junction*. PLoS One, 2012. **7** : e29976.
11. Remédio, L., et al., *Diverging roles for Lrp4 and Wnt signaling in neuromuscular synapse development during evolution*. Genes Dev, 2016. **30**: p. 1058-1069.
12. Yilmaz, A., et al., *MuSK is a BMP co-receptor that shapes BMP responses and calcium signaling in muscle cells*. Sci Signal, 2016. **9**(444): ra87.
13. Punga A.R., et al., *MuSK levels differ between adult skeletal muscles and influence postsynaptic plasticity*. Eur J Neurosci. 2011. 33: p. 890-898.
14. Shin, J., et al., *Single-Cell RNA-Seq with Waterfall Reveals Molecular Cascades underlying Adult Neurogenesis*. Cell Stem Cell, 2015. **17**(3): p. 360-72.
15. Garcia-Osta, et al., *MuSK Expressed in the Brain Mediates Cholinergic Responses, Synaptic Plasticity, and Memory Formation*. J. Neurosci, 2006. **26**(30): p. 7919-7932.
16. Jaime D., et al., *The MuSK-BMP pathway maintains myofiber size in slow muscle through regulation of Akt-mTOR signaling*. Skeletal muscle, 2024. **14**: 1.
17. Fish, L.A., et al., *The MuSK-BMP pathway regulates synaptic Nav1.4 localization and muscle excitability*. biorxiv, 2023. <https://doi.org/10.1101/2023.10.24.563837>
18. Xi, C., et al., *Targeting the MuSK-BMP pathway with antisense oligonucleotides to promote adult hippocampal neurogenesis in neurological and psychiatric disease*. Society for Neuroscience Nanosymposium Abstracts, 2023. Presentation Number: NANO63.05.
19. Fatt, P. and Katz, B., *The electric activity of the motor end-plate*. Proc R Soc Lond Ser B - Biol Sci, 1952. **140**: p. 183–186.
20. Sanes, J.R. and Lichtman, J.W., *DEVELOPMENT OF THE VERTEBRATE NEUROMUSCULAR JUNCTION*. Annu Rev Neurosci, 1999. **22**: p. 389-442.

21. Sanes, J.R. and Lichtman, J.W., *Induction, assembly, maturation and maintenance of a postsynaptic apparatus*. Nat Rev Neurosci, 2001. **2**: p. 791-805.
22. Slater, C.R., *Structural Factors Influencing the Efficacy of Neuromuscular Transmission*. Ann Ny Acad Sci, 2008. **1132**: p. 1-12.
23. Slater, C.R., *The Structure of Human Neuromuscular Junctions: Some Unanswered Molecular Questions*. Int J Mol Sci, 2017. **18**: 2183.
24. Zhang, C., et al., *Ankyrin-dependent Na⁺ channel clustering prevents neuromuscular synapse fatigue*. Curr Biol, 2021. **31**: p. 3810-3819.e4.
25. Wood, S.J. and Slater, C.R., *Safety factor at the neuromuscular junction*. Prog Neurobiol, 2001. **64**: p. 393-429.
26. Winbanks, C.E., et al., *The bone morphogenetic protein axis is a positive regulator of skeletal muscle mass*. J Cell Biol. 2013. 203:345–57.
27. Emde, B., et al., *Wheat germ agglutinin staining as a suitable method for detection and quantification of fibrosis in cardiac tissue after myocardial infarction*. Eur J Histochem., 2014. **58**: 2448.
28. Altman, J., and Das, G.D., *Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats*. Journal of Comparative Neurology, 1965. **124**(3): p.319–335.
29. Doetsch, F., et al., *Subventricular zone astrocytes are neural stem cells in the adult mammalian brain*. Cell, 1999. **97**(6): p. 703-716.
30. Basak, O., and Taylor, V., *Stem cells of the adult mammalian brain and their niche*. Cellular and Molecular Life Sciences, 2008. **66**: p. 1057-1072.
31. Kempermann, G., et al., *More hippocampal neurons in adult mice living in an enriched environment*. Nature, 1997. **386**(6624): p. 493-495.
32. van Praag, H., Kempermann, G., and Gage, F.H., *Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus*. Nature Neuroscience, 1999. **2**(3): p. 266-270.
33. Deng, W., Aimone, J.B., and Gage, F.H. *New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory?* Nature Reviews Neuroscience, 2010. **11**(5): 339-350.
34. Song, J., et al., *Neuronal circuitry mechanism regulating adult quiescent neural stem-cell fate decision*. Nature, 2012. **489**(7414): p. 150-154.
35. Eriksson, P.S., et al., *Neurogenesis in the adult human hippocampus*. Nature Medicine, 1998. **4**(11): p. 1313-1317.
36. Spalding, K.L., et al., *Dynamics of hippocampal neurogenesis in adult humans*. Cell, 2013. **153**(6): p. 1219-1227.
37. Ernst, A., and Frisen, J., *Adult Neurogenesis in Humans- Common and Unique Traits in Mammals*. PLoS Biol, 2015. **13**(1): e1002045.
38. Moreno-Jiménez, E.P., et al., *Adult hippocampal neurogenesis is abundant in neurologically healthy subjects and drops sharply in patients with Alzheimer's disease*. Nature Medicine, 2019. **25**(4): p. 554-560.
39. Boldrini, M., et al., *Human hippocampal neurogenesis persists throughout aging*. Cell Stem Cell, 2018. **22**(4): 589-599.e5.
40. Kempermann, G., et al., *Human Adult Neurogenesis: Evidence and Remaining Questions*. Cell Stem Cell, 2018. **23**(1): p. 25-30.

41. Terreros-Roncal, J., et al., *Methods to study adult hippocampal neurogenesis in humans and across the phylogeny*. Hippocampus, 2023. **33**(4): 271-306.
42. Babcock, K.R., et al., *Adult Hippocampal Neurogenesis in Aging and Alzheimer's Disease*. Stem Cell Reports, 2021. **16**(4): p. 681-693.
43. Malberg, J. E., et al., *Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus*. The Journal of Neuroscience, 2000. **20**(24): p. 9104-9110.
44. Villeda, S. A., et al., *Young blood reverses age-related impairments in cognitive function and synaptic plasticity in mice*. Nature Medicine, 2014. **20**(6): p. 659-663.
45. Terreros-Roncal, J., et al., *Impact of neurodegenerative diseases on human adult hippocampal neurogenesis*. Science, 2021. **374**(6571): p. 1106-1113.
46. Zhou, Y., et al., *Molecular landscapes of human hippocampal immature neurons across lifespan*. Nature, 2022. **607**: 527-523.
47. Bond, A. M., Ming, G. L., and Song, H., *Adult mammalian neural stem cells and neurogenesis: five decades later*. Cell Stem Cell, 2015. **17**(4): p. 385-395.
48. Colak, D., et al., *Adult neurogenesis requires Smad4-mediated bone morphogenic protein signaling in stem cells*. The Journal of Neuroscience, 2008. **28**(2): p. 434-446.
49. Gobeske, K.T., et al., *BMP signaling mediates effects of exercise on hippocampal neurogenesis and cognition in mice*. PLoS One, 2009. **4**(10): e7506.
50. Lim, D.A., et al., *Noggin antagonizes BMP signaling to create a niche for adult neurogenesis*. Neuron, 2000. **28**: 713-726.
51. Mira, H., et al., *Signaling through BMPR-1A regulates quiescence and long-term activity of neural stem cells in the adult hippocampus*. Cell Stem Cell, 2010. **7**(1): 78-89.
52. Diaz-Moreno, M., et al., *Noggin rescues age-related stem cell loss in the brain of senescent mice with neurodegenerative pathology*. PNAS, 2018. **115**(45): p. 11625-11630.
53. Tunc-Ozcan, E., et al., *Hippocampal BMP signaling as a common pathway for antidepressant action*. Cell Mol Life Sci., 2021. **79**(1): 31.
54. Huijbers, M.G., et al., *MuSK myasthenia gravis monoclonal antibodies: valency dictates pathogenicity*. Neurol. Neuroimmunol. Neuroinflamm., 2019. **6**: e547.
55. Takata, K., et al., *Characterization of pathogenic monoclonal autoantibodies derived from muscle-specific kinase myasthenia gravis patients*. JCI Insight, 2019. **4**: e127167.
56. Oh, S., et al., *Precision targeting of autoantigen-specific B cells in muscle-specific tyrosine kinase myasthenia gravis with chimeric autoantibody receptor T cells*. Nature Biotechnology, 2023. **41**: p. 1229-1238.
57. Nascimento, M., et al., *Fractone Bulbs Derive from Ependymal Cells and Their Laminin Composition Influence the Stem Cell Niche in the Subventricular Zone*. J Neurosci, 2018. **38**(16): p. 3880-3889.
58. Daynac, M., et al., *Age-related neurogenesis decline in the subventricular zone is associated with specific cell cycle regulation changes in activated neural stem cells*. Scientific Reports, 2016. **6**: 21505.