Characterization of RHAU Conditional Knockout Mice and the Role of RHAU in Maintaining Post-Synaptic Stability at Neuromuscular Junction

Inauguraldissertation

Zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von

Pu Wu

aus China

Basel, 2011
Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät

auf Antrag von

Prof. Dr. Pico Caroni
(Dissertationsleitung)

Prof. Dr. Silvia Arber
(Korreferat)

Dr. Jan Pielage
(Experte)

Basel, den 24. 05. 2011

Prof. Dr. Martin Spiess
(Dekan)
Table of Contents

General overview ............................................................................................................. 6
Summary .............................................................................................................................. 6
Introduction ....................................................................................................................... 7

Neuromuscular Junction ................................................................................................. 7

NMJ formation: ................................................................................................................. 9
NMJ maintenance .............................................................................................................. 12

Dystrophin and its associated complex in maintaining stability of muscle and
post-synapse at NMJ ........................................................................................................... 15

Dystrophin: ...................................................................................................................... 15
Dystroglycan and the dystroglycan complex: ................................................................. 17
The dystrophin paralog Utrophin: .................................................................................. 19

RNA binding proteins ..................................................................................................... 20

Biochemistry and molecular function of ......................................................................... 20
RNA helicase RHAU/ DHX36 ......................................................................................... 20
RNA binding proteins are known involved in synaptic formation and stability .......... 22
The Fragile X syndrome-causing protein FMRP is involved in synaptic formation
and stability. ................................................................................................................... 23
RNA binding protein Pumilio is involved in synaptic formation ................................... 25

Conditional knockout strategy ....................................................................................... 28
Goal of this thesis ............................................................................................................. 29

Results ............................................................................................................................... 30

Generation of actin-CreER;RHAU$^{+/+}$ inducible Knockout mice in Nagamine
laboratory ......................................................................................................................... 30
Characterization of disease progression pattern upon age-dependent onset ............ 32
Inducible paralytic disorder is independent of motor axon degeneration .......... 38
Motoneuron-specific knockout of RHAU does not cause neuromuscular-
paralytic disorder ............................................................................................................. 44
Muscle-specific RHAU knockout leads to destabilization of AChR cluster at NMJ
............................................................................................................................................ 46
The effect of RHAU loss of function on gene expression at NMJ and non-NMJ regions of muscle fiber

Genes known to be enriched at NMJ
Genes involved in RNA processing and protein synthesis
Genes involved in the cytoskeleton and its binding proteins
Insulin/IGF-1 pathway attenuated in RHAU cKO mice

Endogenous localization of RHAU protein in vitro and in vivo

Discussion

Materials and methods

Animal and genotyping
Immunohistochemistry
Western blot
Antibodies
Axotomy and AAV virus injection
Laser dissection and DNA Microarray
Myoblast culture and differentiation

References

Acknowledgement

Curriculum Vitae
During my PhD thesis study, I mainly focused on characterizing CreER/Loxp-mediated inducible RHAU knockout mice (RHAU cKO mice), which exhibit a progressive paralytic phenotype, and elucidated how loss of the RNA binding protein RHAU leads to paralysis and muscle shrinkage.

I first review some of the literature about the molecules and signaling pathways that have been implicated in neuromuscular junction formation and stability. This includes studies about dystrophin and dystrophin-associated complex, proteins that have an important role in stabilizing postsynaptic acetylcholine receptor clusters at neuromuscular junctions, and may be involved in the defects detected in RHAU muscle-specific knockout mice. The review further includes RNA binding proteins that have been shown to play important roles in regulating synapse formation and function.

The results section includes a detailed characterization of the age-dependent paralytic phenotype in RHAU cKO mice. Then, a motoneuron specific Cre line and Adeno associated virus (AAV) mediated Cre recombinase are employed to dissect the role of motor neuron and muscle in contributing to the paralytic phenotype of RHAU cKO mice. Finally, DNA microarrays are used to probe the effect of RHAU loss of function on transcriptional profiles both at NMJ and non-NMJ regions of muscle fibers.

The thesis closes with a discussion of how a deletion of the RNA binding protein RHAU may lead to neuromuscular disorders.
Summary

The RNA helicase RHAU conditional knockout mice generated in Yoshikuni Nagamine laboratory show neuromuscular-paralytic disorder. However, the mechanisms underlying this neuromuscular-paralytic phenotype caused by RHAU loss of function remains unknown. In this thesis, the goal is to characterize the RHAU conditional knockout mice and to understand the mechanisms of how conditional knockout of RHAU in mice leads to neuromuscular-paralytic disorder.

By inducing RHAU knockout at different ages, we found that mice with RHAU knockout at early stage (3-week-old) die faster with much lower bodyweight than Wildtype mice, while the mice with RHAU knockout at late stage (5-month-old) develop age-dependent progressive neuromuscular paralytic disorder with muscle shrinkage. To understand the synaptic basis of this neuromuscular disorder, the motor axons were genetically labeled with membrane targeted GFP (mGFP). We could not find the denervation of motor axons at NMJs in paralyzed RHAU knockout mice, indicating that the paralytic phenotype caused by loss of RHAU is independent of axon degeneration. To further understand the role of motoneuron in contributing to paralysis observed in RHAU conditional knockout mice, RHAU was specifically deleted in motoneurons by breeding with HB9-cre mice. Consistently, the motoneuron-specific knockout mice show normal motor behavior as WT mice with normal NMJ morphology.

We then knockout RHAU specifically in muscle by expressing Cre recombinase in muscle using Adeno associated virus (AAV). The post-synaptic AChR clusters are destabilized and fragmented into individual boutons upon muscle-specific RHAU knockout. Thus, our results demonstrate that RNA helicase RHAU plays an essential role in maintaining post-synaptic AChR clusters stability at NMJ.
Introduction

Neuromuscular Junction

The mammalian neuromuscular Junction (NMJ) has been widely used as a model to study synapse development and function due to its experimental accessibility. It is the synapse model system that this thesis is focusing on. In mammals, the NMJ is composed of parts of three cell-types: Motor neuron, Schwann cell and Muscle fiber. The motor neuron sends out the cholinergic axons that innervate the muscle fibers. Each adult muscle fiber is only innervated by a single motor axon terminal.

The presynaptic motor terminal is specialized to store and release synaptic vesicles, which contain the neurotransmitter acetylcholine. The postsynaptic site at muscle fibers is highly enriched with acetylcholine receptors (AChRs)(Salpeter and Loring, 1985). The postsynaptic site at muscle fibers is not flat, rather, it is folded. The AChR clusters are on the edge of folds in order to perfectly fit and respond to active zones at presynaptic terminals. The adhesion molecule NCAM and sodium channel are located at the bottom of the junction folds (Covault and Sanes, 1986; Flucher and Daniels, 1989). There are different cytoskeletal molecules also differentially located within the junction fold. Rapsyn and utrophin scaffold proteins are located together with AChR on top, whereas Dystrophin and ankryin are located at bottom of the fold (Covault and Sanes, 1986; Flucher and Daniels, 1989; Peters et al., 1998). The gap between pre- and post-synapse is called synaptic cleft, and is mainly filled with basal lamina matrix. Beside basal lamina, the synaptic cleft contains several signaling molecules (neuregulin and agrin) and acetylcholinesterase (AchE). AchE is involved in rapid degradation of the neurotransmitter acetylcholine after neurotransmitter release.
This figure is adapted from a review published in Neuron by Hall and Sanes, 1993.

The mammalian NMJ is the most studied model for synapse development (Hall and Sanes, 1993), which includes synapse formation, synapse maturation and maintenance. There are still other steps such as elimination of excessive synapses and synapse regeneration upon denervation, which will not be discussed here. The major signaling pathways identified in NMJ underlying the different processes of synapse formation and maintenance will be reviewed next.
NMJ formation:

Synapse formation includes both pre- and post-synaptic differentiation. The post-synaptic differentiation, which is more related to this study, is mainly discussed here. Once the nerve terminal arrives at the myotube, the postsynaptic differentiation starts. To form stable postsynaptic AChR clusters at NMJ, two processes are mainly involved: clustering of diffusely distributed AChRs on myotube membrane, activation of AChR transcription in NMJ-enriched nuclei and repression of transcription in non-NMJ-enriched nuclei in myotube (Hall and Sanes, 1993).

In myotubes, the synthesis of AChR involves several steps: AChR mRNA transcription and translation, and diffuse insertion of AChR in myotube membrane. Once the nerve arrives at the myotube membrane, the diffusely distributed AChRs start to cluster at the site where motor neuron arrives (Anderson and Cohen, 1977; Frank and Fischbach, 1979), which raises the question of what is the signal derived from nerve induces clustering. Several proteins have been isolated (Peng et al., 1991; Peng et al., 1995; Zhou et al., 1997) but only a heparan sulfate proteoglycan protein called Agrin has been implicated in formation of NMJ in vivo. Agrin was initially isolated and cloned from Torpedo electric organ (Nitkin et al., 1987). It is released from motor nerve terminal, and then inserted into basal lamina (McMahan, 1990a; Reist et al., 1992). MacMahan proposed that Agrin is the major cause for postsynaptic differentiation (McMahan, 1990a; Reist et al., 1992). Indeed, both loss of function and gain of function experiments supported this hypothesis. The agrin knockout mice show severe impairment of postsynaptic differentiation with only few neuron-muscle contacts associated with AChR cluster, while AChR clusters are absent at the majority of neuron-muscle contacts (Gautam et al., 1996). Interestingly, the intramuscular nerve branching from motor neuron and presynaptic differentiation are also impaired in agrin mutant mice (Gautam et al.,
In contrast, overexpression of agrin by injecting an agrin expression vector into both innervated and denervated muscle fibers could induce formation of complete postsynaptic apparatus at the site where agrin injected and expressed (Jones et al., 1997). Thus, these studies suggest that agrin is not only necessary but also sufficient to induce postsynaptic differentiation.

However, the agrin hypothesis has been challenged by recent studies that have shown there is pre-patterned AChR cluster localized in the center of muscle fiber before the motor nerve arriving at muscle. This phenomenon was first observed in mice lacking topoisomerase 2β (*top 2β*) (Yang et al., 2000). In this mutant, motor axons are unable to enter within diaphragm and limb muscles. However, the AChR still could cluster in the middle of the muscle fiber, suggesting that the AChR cluster formed in a pre-pattern independent of motor axon innervation (Yang et al., 2000). To further address this question, a similar experiment was done in the HB9 mutant mice, in which the motor neuron progenitor cells lose the ability to differentiate into motor neurons (Yang et al., 2001). In the embryos of HB9 mutant, no muscle fiber innervation is observed, while the AChR cluster is still found in the center of muscle fiber. Interestingly, the pre-patterned AChR clusters formation in the muscle is dependent on a muscle-specific transmembrane kinase called MuSK, since no pre-patterned AChR clusters formed in mice lacking both HB9 and MuSK (Yang et al., 2001). Thus, these studies suggest that the function of neural agrin signal is to refine and stabilize the pre-existing AChR cluster in order to form stable AChR clusters in muscle fiber, rather than to initiate AChR clustering at muscle fiber.

Another question is how agrin acts on myotubes to induce AChR clustering. There must be a receptor and signaling pathways in the muscle part to mediate the effect of agrin derived from motor neuron. Indeed, several molecules have been identified involved in vitro (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994; Martin and Sanes, 1995; Denzer et al., 1997). However, only MuSK kinase has been implicated in AChR clustering in vivo. MuSK kinase is normally restricted within motor endplate and co-localized with AChR clusters, while it is diffusing along the muscle fiber upon nerve denervation (Bowen et al., 1998). MuSK null knockout mice show severe defects.
in NMJ formation (DeChiara et al., 1996). This defect is even more severe than agrin knockout mice, since there is no AChR cluster found in MuSK knockout mice. It has also been demonstrated that agrin failed to induce formation of AChR cluster in the myotubes cultured from MuSK knockout mice (Glass et al., 1996). This failure could be completely restored by re-introducing expression of MuSK. Both in vitro and in vivo evidence strongly suggest that MuSK serves as a critical downstream signaling molecule of agrin, arguing that MuSK may be a receptor for agrin. However, there is no evidence thus far that MuSK could specifically bind to agrin both in vitro and in vivo. Thus, MuSK could be a subunit of the receptor complex responding to agrin. Recently two papers demonstrated that a low-density lipoprotein receptor-related protein 4 (Lrp4) could bind to MuSK to form clusters (Kim et al., 2008; Zhang et al., 2008a), moreover, Lrp4 could directly bind to agrin as agrin co-receptor. Mice lacking Lrp4 display a similar defect of NMJ formation as MuSK Knockout mice: absence of AChR clusters and aberrant presynaptic branching (Weatherbee et al., 2006). These studies strongly suggest that Lrp4 is the long-sought agrin receptor.

The function of the Agrin/Lrp4/MuSK/AChR axis in NMJ formation seems to be supported by a number of different evidences. The next question raised is that what is the signaling molecule downstream of this axis. Several candidate molecules have been identified. One of them is rapsyn, which is a cytoplasmic protein specifically co-localized with AChR at NMJ (Noakes et al., 1993). Similar with MuSK knockout mice, no AChR clusters form in rapsyn knockout mice (Gautam et al., 1995), suggesting that rapsyn is essential for NMJ formation and also placing rapsyn in the same pathway with MuSK. Interestingly, MuSK still can form clusters in rapsyn deficient mice, suggesting that MuSK is an upstream molecule of rapsyn.

Another protein called Tid-1 that interacts with MuSK was identified by a yeast two-hybrid screen (Linnoila et al., 2008). Tid-1 is a mammalian homolog of Drosophila Tumorous imaginal discs that co-localizes with AChR at both developing and adult NMJ. Knocking down Tid-1 in skeletal muscle by using shRNA leads to dispersed AChR cluster (Linnoila et al., 2008), suggesting that Tid-1 is essential for maintaining postsynaptic stability at adult NMJ. Knockdown
of Tid1 in cultured myotube prevents both spontaneous and agrin-induced formation of AChR, whereas the agrin-induced clustering of MuSK is not affected by Tid1 reduction (Linnoila et al., 2008), strongly suggesting that Tid1 is a downstream factor of MuSK in the formation and maintenance of AChR clusters at NMJ.

Dok-7 is a cytoplasmic protein preferentially expressed in skeletal muscle and heart (Okada et al., 2006). In particular, Dok-7 is specifically interacting with the cytoplasmic domain of MuSK at NMJ. Expression of Dok-7 suppressed by siRNA in myotube culture leads to decreased MuSK and AChR phosphorylation (Okada et al., 2006), indicating that Dok-7 is important for MuSK activation. Indeed, the MuSK-dependent spontaneous formation of AChR cluster is inhibited upon siRNA mediated Dok-7 inhibition. Furthermore, the agrin-dependent AChR cluster is also impaired upon Dok-7 inhibition (Okada et al., 2006). These results indicate that Dok-7 is required for both spontaneous and agrin-dependent AChR cluster formation in vitro. The phenotype of Dok-7 knockout mice is very similar with MuSK knockout mice, showing both pre- and post-synaptic abnormalities (Okada et al., 2006). There is no detectable AChR cluster in Dok-7 mutant mice even at E14.5 (Okada et al., 2006), when the nerve and agrin-independent AChR cluster is supposed to be formed. These data demonstrate that Dok-7 is essential for NMJ formation both in vitro and in vivo.

**NMJ maintenance**

Compared to NMJ formation, less is known about NMJ maintenance in adult, especially about the molecules and signaling pathways required for the late stage of NMJ maintenance. The most studied molecules involved in this process are dystrophin-glycoprotein complex (DGC) that links muscle cytoskeleton to extracellular matrix of muscle cells (Grady et al., 2000). It includes dystrophin
and its homolog utrophin, the three transmembrane proteins dystroglycan, sarcoglycan and sarcospan, and two soluble protein dystrobrevins and syntrophins, which are reviewed extensively in another introduction part.

The DGC complex is distributed over the surface of muscle fibers, both at synaptic and non-synaptic regions. The DGC has been implicated in NMJ formation in vitro since agrin released by nerve terminals could bind alpha-dystroglycan (Campanelli et al., 1994; Gee et al., 1994). Alpha-dystroglycan loss of function attenuates agrin-induced clustering of AChR (Campanelli et al., 1994). There is however no evidence suggesting that DGC is involved in NMJ formation in vivo since knockout of almost any gene in DGC complex in mice does not cause severe NMJ formation defect (Lyons and Slater, 1991; Grady et al., 1997a; Duclos et al., 1998; Hack et al., 1998). Knockout of either Utrophin or Dystrophin leads to only subtle postsynaptic abnormality (Lyons and Slater, 1991; Grady et al., 1997a). Mice lacking alpha- or gamma-sarcoglycan have normal NMJ. (Duclos et al., 1998; Hack et al., 1998). Alpha-dystrobrevin is a cytoplasmic protein that localizes to sarcolemma, but also concentrates at NMJ. Mice lacking alpha-dystrobrevin show a mild muscular dystrophy phenotype but normal NMJ formation (Grady et al., 1999). These studies demonstrate that DGC is not essential for NMJ formation in vivo.

Interestingly, in the myotubes cultured from alpha-dystrobrevin knockout mice, agrin could induce AChR clustering in absence of alpha-dystrobrevin, while the AChR cluster starts to rapidly destabilize upon removal of agrin (Grady et al., 2000), suggesting that dystrobrevin is specifically required for AChR cluster maintenance but is not for initially NMJ formation. This result not only provides an entry point to study the mechanism underlying NMJ maintenance, but also suggests the distinct mechanisms underlying regulation of NMJ formation and maintenance.

Further study has been carried out to address the relationship between alpha-dystrobrevin and other components of DGC complex. Myotubes cultured from dystroglycan deficient mice have normal AChR cluster formation with or without agrin treatment, suggesting that dystroglycan is not required for NMJ formation
either (Grady et al., 2000). Further looking at the sarcolemma in muscle fibers of dystroglycan knockout mice indicates that there is no sarcolemma associated DGC complex formed in this animal (Henry and Campbell, 1998). Interestingly, the ability to form AChR cluster in response to agrin is not impaired either, suggesting that the whole DGC complex is actually dispensable for the postsynaptic agrin signal mediating NMJ formation, but essential for maintaining NMJ stability.

Another molecule whose signaling has been implicated in maintaining AChR cluster stability is tyrosine receptor kinase B (TrkB) (Gonzalez et al., 1999b). Neurotrophins and their receptors have been shown to play a role in regulating dendritic outgrowth, remodeling, and stability in cortical slice culture (McAllister et al., 1995; Horch et al., 1999), while its role at neuromuscular synapse has not been well addressed. One study showed that both the full-length and truncated forms of trkB are located at the synaptic-enriched region of muscle fibers with innervation and denervation (Gonzalez et al., 1999a), suggesting that trkB may play an important role in NMJ synaptogenesis or NMJ stability. Indeed, disrupting the trkB signaling in muscle fiber by over-expressing dominant-negative truncated trkB leads to disassembly of AChR clusters at both neonatal and adult NMJ, indicating that trkB signaling is essential for keeping stability of AChR clusters. This is further supported by the evidence that fragmentation of AChR clusters is observed at the NMJs from trkB⁺⁻ mice, in which the trkB protein level is only half of wild type mice. Since it is believed that there is constant information exchange between motor neuron nerve terminal and muscle fiber, namely, the neurotrophic factors released from muscle fiber retrogradely transported and taken up by presynaptic terminal, which in turn facilitates the presynaptic release (Oppenheim, 1996). Thus, it is unclear whether motor neuron and schwann cell are involved in regulating AChR cluster stability mediated by trkB signaling as well. This has been studied in the cultured myotubes. The agrin-induced AChR clusters in myotubes are also disrupted by overexpressing truncated trkB, showing punctate and fragmented AChR cluster. This in vitro study demonstrates that the trkB signaling could regulate the postsynaptic stability at NMJ in absence of nerve terminal and schwann cell,
indicating that the signaling pathways in muscle are sufficient to maintain its AChR cluster stability.

In addition to AChR cluster stability, AChR density at NMJ is also regulated by a genetic program. A gene called ARIA (acetylcholine receptor-inducing activity) has been shown to be involved in this process (Sandrock et al., 1997). ARIA is a member of neuroregulin family ligands for transmembrane receptor tyrosine kinases including ErbB2, ErbB3, and ErbB4 (Falls et al., 1993). ARIA was originally extracted from brain lysates and identified as a potent activator of AChRs synthesis in cultured myotubes (Usdin and Fischbach, 1986). ARIA is abundant in both developing and adult motor neuron nerve terminal, and its receptors are present at the membrane of skeletal muscle (Sandrock et al., 1995). ARIA is particular enriched at developing motor neuron nerve terminal and could specifically accumulate in the basal lamina of NMJ clefts (Goodearl et al., 1995). The subtype of AChR synthesis induced by ARIA is AChR containing ε subunit, which replaces the γ subunit during NMJ development (Martinou et al., 1991), suggesting that ARIA is involved in NMJ maturation.

Interestingly, a significant reduction of postsynaptic AChR density is observed at adult NMJ in the ARIA heterozygous knockout mice, which is partially lacking the isoform containing immunoglobulin-like domain (Sandrock et al., 1997). This is further supported by electrophysiology study showing that significant reduction of amplitude of spontaneous miniature membrane end plate potential. This study demonstrates that post-synaptic density of AChR is maintained by a genetic program such as ARIA as well.

Dystrophin and its associated complex in maintaining stability of muscle and post-synapse at NMJ

Dystrophin:
The positional clone of mutated dystrophin in X-chromosome as a cause of muscular dystrophy provided an entry point to study its underlying molecular mechanisms (Koenig et al., 1987b). Dystrophin is one of the longest genes identified at DNA level so far, which consists of 79 exons covering 2.5 megabases at locus Xp21 in the genome (Koenig et al., 1987a; Coffey et al., 1992; Monaco et al., 1992a). The full-length 14 kb mRNA was found to predominantly expressed in skeletal and cardiac muscle with lower level in brain (Koenig et al., 1987a). The amount protein encoded by this gene is decreased or lacking in muscular dystrophy patients (Koenig et al., 1987a).

Dystrophin is a 427 kDa cytoskeletal protein belonging to the beta-spectrin/alpha-actinin protein family (Koenig et al., 1987a), which is featured with its N-terminal actin binding domain followed with variable number of spectrin-like repeat domain (Winder and Gibson, 1995; Winder et al., 1995; Rybakova and Amann, 1996; Rybakova et al., 1996). Dystrophin is a rod-shaped cytoplasmic protein and an important part of a protein complex that connects the sarcolemma cytoskeleton of a muscle fiber to the surrounding extracellular matrix through the muscle membrane (Campbell and Kahl, 1989), thus dystrophin connects the actin cytoskeleton to the dystrophin-associated protein complex in the sarcolemma, which may play an important role in stabilizing the muscle fiber membrane.

Dystrophin deficient mice (or called mdx mice) has elevated serum creatine kinase level and exhibits some clinical characteristic of muscular dystrophy(Bulfield et al., 1984). This study not only confirmed that dystrophin loss of function is the cause of muscular dystrophy, but also provides an animal model for studying pathology. However, the phenotype of mdx mice is not dramatic. The muscle weakness is not obvious and life span is not grossly reduced in mdx mice (Lynch et al., 2001b). Interestingly, hypertrophy is the feature of mdx mice, although it doesn't happen in muscular dystrophy patient. Based on in vivo study, the normalized force output is reduced (Lynch et al., 2001a). Muscle fiber degeneration was observed at round 3-4 week – old, which followed by its regeneration (Coulton et al., 1988). From then on, muscle fiber degeneration and regeneration constantly happen in the adult mdx mice.
Dystroglycan and the dystroglycan complex:

Since dystrophin has been found enriched in muscle membrane fraction, a number of membrane protein were identified to be associated with dystrophin, which were termed dystrophin-associated protein complex (DPC) (Campbell and Kahl, 1989; Yoshida and Ozawa, 1990). The first component of DPC identified is dystroglycan, a gene that only consists of two exons (Ibragimov-Beskrovnaya et al., 1993). The product of this gene is further processed by an unknown protease to produce alpha- and beta- dystroglycan. In muscle, alpha-
dystroglycan is a 156 kDa protein located in the extracellular matrix, whereas beta-dystroglycan is a 43 kDa protein that has single transmembrane domain inserted into the muscle plasma (Smalheiser and Kim, 1995).

Dystroglycan was shown to be involved in a variety of cellular functions. In muscle, it has been demonstrated that dystroglycan is involved in formation of neuromuscular junction (NMJ). Alpha-dystroglycan has been shown to bind directly to secreted glycoprotein agrin, which initiates the clustering of postsynaptic acetylcholine receptor (AChR) in vitro (Campanelli et al., 1994; Gee et al., 1994). This study leads to the hypothesis that alpha-dystroglycan is the receptor for agrin. However, further study using myotube cultured from alpha-dystroglycan knockout mice showed that deletion of alpha-dystroglycan does not prevent the agrin-induced AChR clustering. Thus, the function of DPC in synaptogenesis is probably stabilizing the formed cluster of AChR rather than initiating AChR clustering. This is supported by the evidence that components of DPC including utrophin and alpha-sarcoglycan are recruited to AChR cluster after agrin induction (Campanelli et al., 1994).

Specific deletion of dystroglycan in muscle causes progressive muscular dystrophy (Cote et al., 1999). However, the muscle membrane and its extracellular matrix formation are not impaired, suggesting that extracellular matrix formation is independent of dystrophin-associated protein complex. Muscle from this chimeric mice degenerate in response to activity-dependent mechanical injury. The dystrophin and alpha-sarcoglycan are significantly reduced at the protein level both at the sacrolemma and the NMJ (Côté et al., 1999). The NMJ in chimeric mice shows abnormal morphology and severe reduction in the level of acetylcholinesterase. Thus, these results further suggest that the role of dystroglycan and DPC play an important role in synaptic differentiation and stability.
The dystrophin paralog Utrophin:

Utrophin is a gene cloned after dystrophin with very similar cDNA sequence to dystrophin, with only distinction at C-terminal (Love et al., 1989). Its full-length cDNA is 13kb long and encodes a protein with molecular mass of 395kDa. The protein structure of Utrophin is predicted as similar to dystrophin (Tinsley et al., 1992).

Utrophin is much more ubiquitously expressed than dystrophin, which only expresses in skeletal, cardiac, and smooth muscle (Pons et al., 1994). Interestingly, in developing muscle, utrophin is found all along the sarcolemma while in adult normal muscle, it is restricted to the NMJ and myotendinous junctions (Nguyen et al., 1991). Like the distribution of AChR, utrophin is also found at crests of the junction folds, where utrophin is co-localized with AChR clusters in both developing muscle and muscle culture (Bewick et al., 1996).

To test whether there is functional redundancy between dystrophin and utrophin, several lines of mdx mice harboring muscle-specific utrophin transgenes have been generated (Tinsley et al., 1996a; Tinsley et al., 1998). In these transgenic lines, the utrophin is localized along sarcolemma, and the DPC are reconstituted. Surprisingly, the muscle pathology caused by lack of dystrophin is significantly reduced by over-expressing utrophin (Tinsley et al., 1996a). These studies not only suggest that utrophin and dystrophin are functionally redundant, but also may provide a therapy for muscular dystrophy patients.

Double knockout of both dystrophin and utrophin causes much more dramatic muscular dystrophy phenotype than knockout either protein in isolation (Grady et al., 1997b). The double mutant mice lose weight, develop earlier muscle fiber necrosis and spinal deformities, and die very young, which recapitulates most muscular dystrophy phenotypes. These studies further suggest that utrophin is functional redundant with dystrophin.
RNA binding proteins

Biochemistry and molecular function of RNA helicase RHAU/ DHX36

Protein expression level could be controlled post-transcriptionally mainly through regulating either mRNA stability or mRNA translation. Stability of mRNA is dynamically regulated by a variety of signals on specific sequence of RNA molecule (Wilusz et al., 2001). The most common sequence among the cis-acting instability element identified so far is the AU-rich element (ARE) located in the 3 UTR of unstable mRNAs (Shaw and Kamen, 1986). Many mRNAs with short half-lives such as those encoding cytokines and growth factors have been shown to have an ARE element. A number of ARE binding proteins have been isolated to regulate the ARE-containing mRNA stability (Wilusz and Wormington, 2001). These protein could either promote ARE mRNA decay or increase the stability of ARE mRNA.

RHAU (RNA helicase associated with AU-rich element) or called DHX36 was identified as an ARE binding protein that directly binds to the ARE of urokinase plasminogen activator (uPA) mRNA together with other ARE binding protein HuR and NFAR1 (Tran et al., 2004).

Analysis of RHAU protein sequence suggests that RHAU belongs to the DExH/D family of ATP-dependent RNA helicase. It contains evolutionarily conserved RNA helicase core regions. Two RHAU isoforms have been cloned. A longer isoform is identical to the sequence of MLEL1, which has been published before (Kuroda et al., 1991), and the other isoform is lacking 14 amino acids within the core RNA helicase region, which termed RHAUΔ14. The cellular localization of the two RHAU isoforms is distinct. RHAU is preferentially localized in nucleus, while the RHAUΔ14 is mainly distributed in the cytoplasm. This suggests that the sequence of lacking 14 amino acids contains nuclear localization signal. Both isoforms are functional in regulating mRNA stability, since over-expressing both isoforms are
able to accelerate the degradation of endogenous uPA mRNA, while down-regulation of both isoforms could increase the stability of uPA mRNA. The ATPase domain of RHAU is required to exert its function of mRNA destabilization, since the ATPase defective RHAU is not able to enhance the mRNA decay (Tran et al., 2004).

Biochemical analysis of RHAU in HeLa cell line indicates that the nucleus shows higher expression level of RHAU than cytoplasm (Iwamoto et al., 2008), suggesting that RHAU may also play an important role in transcription in addition to its cytoplasmic role in mRNA degradation. RHAU is concentrated in nuclear speckles composed of splicing factors and mRNAs but less distributed with heterochromatin, suggesting that RHAU is more closely associated with nuclear RNA. When cells treated with ActD, a transcription inhibitor to induce transcriptional arrest, RHAU is no longer enriched in the nuclear speckles but shifted to be around nucleoli (Iwamoto et al., 2008). The transcriptional arrest induced structures of RHAU resembled the nucleolar caps around nucleoli in mammals. This structure of RHAU is closely co-localized with RNA helicase p68 and p72, suggesting that RHAU is involved in the transcription-related RNA metabolism. Moreover, the mRNAs, whose steady-state level is affected by RHAU depletion in HeLa cells, do not show any significant change in their half-lives, measured by microarray (Iwamoto et al., 2008). This study strongly suggests that these transcripts are subjected to transcriptional regulation.

Environmental stress like heat shock, oxidative stress triggers a sudden translational arrest, causing a rapid polysome disassembly. This event activates a process in which mRNA from disassembling polysomes is sorted and the individual transcript is determined. Many mRNAs and RNA binding proteins involved in RNA metabolism are recruited to cytoplasmic organelles called stress granules. When treated HeLa cells treated with well-know stress granule inducer arsenite, the accumulation of RHAU was observed in a distinct cytoplasmic location that co-localized with stress granule marker TIA-1, suggesting that RHAU is a novel stress granule associated protein (Chalupníková et al., 2008). Further biochemical analysis indicates that RHAU could directly interact and bind to RNA in vivo via its N-terminal RNA-binding domain. This RNA binding
domain is essential and sufficient for localizing RHAU to stress granules since over-expressing N-terminal RNA-binding domain alone fused with GFP could still co-localize with stress granule marker (Chalupníková et al., 2008). Thus, RHAU is identified as the fourth RNA helicase after rck/p54, DDX3, and eIF4A that could recruit to stress granule upon cells under stress.

RHAU was also independently discovered as a resolvase for G4-DNA (Vaughn et al., 2005). G4-DNA is a highly stable alternative DNA structure resulting from the propensity of guanine-rich sequences of DNA and RNA to form an atypical and thermodynamically stable four-stranded helical structures. G4 structure in vivo could be relevant to impairment of DNA repair, transcription and translation initiation. G4 structure has also been demonstrated to be involved in immunoglobulin genes rearrangement, promoter activation and telomere maintenance. Since G4-DNA structure is involved in a variety of physiological processes, its resolution must be regulated. The recombinant helicase RecQ is the only enzyme that could resolve the tetramolecular G4-DNA into single stranded so far. The G4-DNA resolving activity of RHAU was captured in HeLa cell lysates with high robustness and specificity in ATP-dependent manner (Creacy et al., 2008). Biochemical analysis demonstrated that the Amino-terminal of RHAU is essential and sufficient to bind the G4 structure (Lattmann et al., 2010). Further analysis indicates that there is an evolutionarily conserved region within amino-terminal termed RHAU specific motif that determines the affinity and binding specificity.

**RNA binding proteins are known involved in synaptic formation and stability**

RNA binding proteins (RBP) plays the crucial role in RNA metabolism. They are involved in almost all steps of RNA biogenesis from pre-mRNA splicing, RNA maturation, RNA transport, translation and degradation (Burd and Dreyfuss, 1994). RBPs can bind to both coding RNA and non-coding RNA to form a complex called ribonucleoprotein (RNP), which enables RNA to remain stable until being
transported to the location where translation occurs (Shyu and Wilkinson, 2000).

RBPs are considered to be particularly crucial for maintaining functional synapse and its plasticity in polarized neuron due to the long distance from cell body to the synapses, where local translation happens (Schuman, 1999). Neurodegenerative diseases are the major clinical manifestation when RBP-mediated RNA processing function impaired (Bassell and Kelic, 2004). There are several RBPs that have been identified to play important roles in synaptic function are selectively reviewed here.

The Fragile X syndrome-causing protein FMRP is involved in synaptic formation and stability.

Fragile X syndrome is a most common hereditary x-linked mental retardation in human. Affected patient manifests hyperactive and autism-like behavior with cognitive deficits. The syndrome is caused by more than 200 CGG triplets located within the 5-UTR of FMR1, which is an RNA binding protein (Bardoni and Mandel, 2002). The hypermethylation of CpG island causes gene silencing, which leads to absence of FMRP protein in the patient (Jin and Warren, 2000). Thus, fragile X syndrome is caused by RNA binding protein loss of function.

FMRP is expressed in many tissues and particularly abundant in the neuron, where FMRP is cytoplasmic protein present in RNPs that associate with polyribosomes both at soma and synapse in neuron (Khandjian et al., 1996), which suggesting that its involvement in RNA post-transcriptional regulation. It has been demonstrated that polyribosomes are often located within dendrite and are concentrated at dendritic spines (Steward and Schuman, 2001; Zalfa et al., 2006; Zalfa and Achsel, 2006). This observation suggests a working model that FMRP binds to specific mRNA to form RNPs, and then transport to synapses followed with local translation upon appropriate stimulus. Various approaches have been used to acquire evidence to support this hypothesis. First of all, the
functional targets of FMRP have to be defined. There were two papers in 2001 trying to elucidate the targets of FMRP by using immunoprecipitation followed by microarray (Brown et al., 2001; Darnell et al., 2001). These study identified a number of FMRP binding mRNA that are involved in neuronal development and synaptic function in the rat brain extract. These mRNAs include MAP1B, Arc and CaMKIIα, which are well-known dendritically localized mRNA. Interestingly, the FMRP specifically binds to the sequence that tends to form G quartet structure (Darnell et al., 2001). At the same time, the mRNAs associated with polyribosome are also purified and then analyzed with microarray. Nearly 50% mRNA in polyribosome are also found in the FMRP associated RNPs (Ceman et al., 1999). Thus, these studies strongly suggest that FMRP is involved in local protein synthesis at synapse. The other question is how the mRNA associated with FMRP transported to the dendrite from cytoplasm. Interestingly, the RNA granules associated with FMRP were observed to distribute to a F-actin-rich compartment including filopodia, spines and growth cones during hippocampal neuron development (Togel et al., 1998; Miyashiro et al., 2003; Dictenberg et al., 2008). The level of FMRP and its targeted mRNAs within dendrite are reduced by disrupting microtubule dynamics, which suggests that the transportation of FMRP associated RNAs requires and depends on microtubules (Togel and Wiche, 1998; Antar et al., 2005a).

The question raised is that how the absence of protein involved in local protein synthesis causes the mental retardation syndrome. To answer this question, FMRP knockout mice have been generated to model fragile x syndrome in vivo. Interestingly, immature long and thin dendritic spines were observed in the slice culture of barrel cortex of FMRP KO mice from postnatal day 7 to postnatal day 14, which is overlapping with the critical period (Nimchinsky et al., 2001). In contrast, for the dissociated 3-week-old hippocampal neurons of FMRP KO mice, short and low-density spines were observed (Antar et al., 2005b). The two studies suggest that FMRP could involve in the process both for synaptogenesis and spine maintenance, and suggests that abnormal spine morphology may underlie the mechanism of how loss of FMRP leads to cognitive deficit.
Synaptic plasticity is associated with change of dendritic spine morphology. Interestingly, the enhanced mGluR-dependent LTD was observed in the hippocampal slice culture of FMR1 KO mice (Huber et al., 2002). mGluR5 induced protein synthesis dependent LTD is based on postsynaptic local protein synthesis (Snyder et al., 2001). Moreover, it also has been shown that enhanced mGluR5 dependent LTD is associated with elimination of synapse and longer dendritic spine. Thus one mechanism could be that FMRP regulates its mRNA targets that are involved in mGluR-dependent LTD. The absence of FMRP leads to enhanced mGluR-dependent LTD, synapse elimination and increased filopodia, which eventually affects the learning and memory in FMR1 KO mice.

The FMRP protein is the example of how a gene mutated in neurological diseases with RNA binding activity leads to discovery of its role in synaptic formation and function. By generating and using the genetic animal model, it not only makes us understand more about mechanism underlying neurological diseases, but also gain more information about basic question such as synaptic formation and function.

**RNA binding protein Pumilio is involved in synaptic formation.**

Pumilio was also initially identified as a maternal gene involved in transporting the abdominal signal during drosophila embryo development (Murata, 1995). Pumilio acts as a translational repressor for maternally supplied hunchback mRNA progressively toward to posterior of embryo in order to create the hunchback concentration gradient throughout embryo (Murata, 1995). The hunchback gradient subsequently leads to regulation of downstream genes for the formation of segmental pattern of embryo.

Pumilio is an evolutionarily conserved RNA binding protein belong to PUF domain containing protein family. It binds to NRE sequence 3-UTR region of hunchback mRNA to form a pum-RNA complex that then recruits additional factors Nanos and Brain Tumor to block the Hunchback mRNA translation.
Recently it has been shown that Pumilio is also involved in Drosophila germline development (Asaoka-Taguchi et al., 1999). The feature of transcriptional quiescence, mitotic arrest and migration to the somatic gonadal site for embryonic germline cell ensures the proper development of the germline. In Drosophila pumilio mutant, pumilio deficiency leads to disruption of germline cells transcriptional quiescence, which is indicative of premature expression of germline specific marker PZ198 (Forbes and Lehmann, 1998). Moreover, the premature mitosis and migration defect were observed in the migrating pole cells. The translation of Cyclin B messenger RNA is enhanced upon the pumilio loss of function, suggesting that Pumilio functions as a translation repressor.

For nervous system, Pumilio was initially identified together with RNA binding protein staufen in a genetic screen in search for genes involved in long-term memory formation in Drosophila (Dubnau et al., 2003). Other genes such as Drosophila homolog oskar, eIF5C mutant were also found with long-term memory deficit. This study suggests that transport and localized translation of newly transcribed mRNA are important for forming long-term memory. Since synaptic plasticity is thought to be the biological basis for learning and memory, many studies were carried out to address the role of Pumilio in the synaptic development and plasticity.

Dendritic arborization neuron in Drosophila peripheral nervous system was used to address the role of Pumilio in dendritic morphogenesis (Ye et al., 2004b). Pumilio and Nos are both found expressed in the neuron and its dendrites in Drosophila. At dendrites, Pumilio and Nos are specifically co-localized with the RNA granules, which are visualized by Syto-14 dye. This suggests that Pumilio/Nos complex is probably also involved in the dendritic translation repression. To directly test if Pumilio/Nos complex is participated in dendritic morphogenesis, either Pumilio or Nos was over-expressed or ablated in the dendritic arborization neuron. Interestingly, the high-order dendritic branch is reduced in Class III and IV dendritic arborization when the Pumilio/Nos are over-expressed. Again specifically observed in Class III and IV dendritic arborization, they show abnormal dendrite morphology upon ablation of Pumilio/Nos. This study demonstrates that Pumilio and Nos are essential for
dendritic morphogenesis in a cell-type specific manner. In addition, over-expressing a mutant form of pumilio that is defective in binding to hunchback mRNA in those vulnerable neurons could still produce the same defect in dendritic morphogenesis as the over-expression of wildtype form. This suggests that binding to hunchback mRNA to suppress its translation is not required for Pumilio/Nos complex to control dendritic morphogenesis.

Distribution of Pumilio protein is mainly cytoplasmic in most larval Drosophila neuron. In muscle, Pumilio is localized to the region surrounding larval NMJ boutons. The NMJ boutons are larger and fewer in number in absence of Pumilio, while increased expression of Pumilio leads to decreased bouton size and increased bouton number (Menon et al., 2004a). Thus pumilio is also involved in regulation of NMJ formation in Drosophila. Interestingly, the GluRIIa glutamate receptor is up-regulated in the Pumilio mutants, which suggesting that Pumilio also functions as a translational repressor. Indeed, the aggregates of translation factor eIF-4E were increasingly accumulated at postsynaptic site at NMJ in Drosophila Pumilio mutant (Menon et al., 2004a). Further study also showed that Pumilio binds directly to 3 UTR of eIF-4E mRNA. Thus, Postsynaptic Pumilio regulates the synaptic function by directly control eIF-4E expression.

A line of drosophila mutant called bemused was isolated as it exhibits increased neuronal excitability (Schweers et al., 2002). This mutant is due to insertion of P-element into Pumilio locus, which leads to loss of function of Pumilio gene. By overexpressing Pumilio in motoneuron of Drosophila, it causes decreased motoneuron excitability, which is opposite of Pumilio loss of function (Schweers et al., 2002). One of the possible mechanisms of how Pumilio regulating excitability is through translationally repressing gene encoding voltage-dependent sodium channel. A recent study showed that Pumilio could directly bind to and represses translation of paralytic (para) mRNA encoding Drosophila sodium channel (Murata and Wharton, 1995). This repression requires cofactors Nanos and Brat. Thus, Pumilio is not only involved in regulation of NMJ formation, but it also involved in controlling motoneuron excitability.
Pumilio targeted RNAs have been identified by using both computational and experimental methods (Chen et al., 2008). Bioinformatic approach has been undertaken to first systematic search for mRNAs specifically bind to Pumilio via specific NRS (Nanos Response Sequence) sequence in 3-UTR of mRNA. A number of new synaptic -enriched genes including Dlg1, a drosophila homolog of PSD-95 were identified together with previous known genes like Hb, Bcd, cyB. Further experiment conformed the informatic prediction. The second approached used is to precipitate the mRNAs binding to Pumilio, followed with the DNA microarray (Gerber et al., 2006). This study identified hundreds of mRNAs that are associated with Pumilio at different embryonic development stages. Interesting to note that many of the genes identified are functionally related, which suggesting that functionally related genes are coordinated by the same RNA binding protein.

**Conditional knockout strategy**

Conventional knockout gene targeting technique in mice provided a great opportunity to study gene function in a post-genomics era. However, conventional knockout of many genes leads to embryonic lethality, which makes it is almost impossible to understand the role of these genes in adult animals. The reason why knockout of many genes causes embryonic lethality is because these genes are important for certain tissue development. Thus, to address the tissue-specific function or the role of genes in adult, a tissue-specific or inducible knockout method has to be developed.

Cre/LoxP system could elegantly circumvent this problem by knocking out these genes in a tissue specific manner, or in an inducible manner. Thus, it is a very popular technique to study the gene function in adult such as age-dependent diseases and learning and memory. The Cre recombinase could be driven by any tissue specific promoters in order to achieve the tissue specific recombination (Tsien et al., 1996). To knockout genes in an inducible manner, the Cre recombinase could be further fused with nuclear receptor Estrogen Receptor (CreER), so that the Cre only goes inside the nucleus to induce DNA
recombination up treating with Estrogen receptor ligand (Feil et al., 1996). By doing this, the targeted genes could be knocked out in any tissue at any time.

Since conventional null knockout of RNA helicase RHAU leads to embryonic lethality, in order to understand the role of RHAU in adult animal, the RHAU conditional knockout mice are generated in Nagamine laboratory. The RHAU exon 8 encoding ATPase, which is essential for RHAU activity, is floxed with LoxP sites. The RHAU floxed mice were bred with transgenic mice expressing CreER under actin promoter to knockout RHAU in an inducible manner. Interestingly, a paralytic phenotype was observed in mice with RHAU knockout in adult. This suggests that the RNA helicase RHAU may play an important role in motor function in mice.

**Goal of this thesis**

This thesis is trying to systematically characterize RHAU conditional knockout mice and elucidate the mechanism underlying the paralytic phenotype. By doing this, it would not only provide information for establishing an inducible animal model for studying how RNA processing defects contribute to paralytic disorder, but also could provide insight into the physiological and molecular function of RNA helicase RHAU.
Results

Generation of actin-CreER;RHAU\textsuperscript{fl/fl} inducible Knockout mice in Nagamine laboratory

Since conventional null RHAU knockout is embryonic lethal in mice (unpublished result from Nagamine lab), which makes it is difficult to analyze the function of RHAU in adult. To circumvent this, conditional RHAU knockout (RHAU cKO) mice were generated by breeding RHAU exon 8 floxed mice with transgenic mice harboring Cre fused with Estrogen receptor (CreER) under control of Actin promoter. Global knockout could be achieved by injecting Tamoxifen in a dose of 10mg per 10g body weight at any age (Figure 1a). 10-week old mice were initially injected to look at the effect of RHAU knockout on adult mice. After 1 month after first tamoxifen injection (10-day injection), the mice were sacrificed in order to check the knockout efficiency by PCR and western blot. The genomic DNAs were extracted from different tissues of Knockout induced mice, and then PCR was performed to check the Cre-mediated recombination through amplifying the Loxp flanked region. The result indicates that tamoxifen-induced recombination occurs throughout different tissues (Figure 1b). The RHAU protein level was also checked by using western blot with a RHAU monoclonal antibody recognizing 991-1007 amino acid in the C-terminal of RHAU. The dramatic knockdown of protein level was observed throughout the body (Figure 1c). These results demonstrate that the efficient RHAU conditional knockout could be achieved by creER/loxp system.
a)  

![Diagram showing gene regulation with Actin promoter, CreER\(^T\), LoxP, RHAU, and LoxP](image)

**Injecting Tamoxifen**

1mg/10g tamoxifen

b)  

![DNA gel electrophoresis image](image)

- Heart
- Muscle
- Colon
- Brain
- Lung
- Skin
- Kidney
- Stomach
Figure 1: Generation of RHAU conditional knockout mice a) Strategy of generation CreER/Loxp mediated RHAU inducible knockout mice. b) Confirmation of RHAU knockout by PCR in different tissues. The arrow indicated band represents that the floxed RHAU was excised by injecting tamoxifen. c) RHAU knockout efficiency determined by western blot in different tissues using RHAU monoclonal antibody recognizing 991-1007 amino acid in the C-terminal of RHAU. Upper panel is the blot for RHAU protein, and the lower panel is the blot for actin housekeeping control.

**Characterization of disease progression pattern upon age-dependent onset**

By taking advantage of the inducible knockout system, we could knockout RHAU at any age. We injected tamoxifen into both three-week and five-month old mice in order to look at the effect of age on RHAU knockout effect in mice. For three-week old mice, we found that the mice starts to be sick around four- or five-day after first injection (Figure 2a). The mice look very weak with reduced body weight, while for five-month-old injected mice, they start to develop the symptom with hindlimb clasping around one month after first injection (Figure 2b). The symptom progresses rapidly from then on. Some mice start to show a paralytic phenotype and reduced body weight within two weeks (Figure 2c). When we sacrificed a animal with a severe phenotype, we found the triceps surae muscle in the hindlimb showed dramatic shrinkage (Figure 2d). Thus, these observations strongly suggest that the mice develop a neuromuscular disorder with muscle dystrophy.
To systematically analyze the disease onset and progression pattern in RHAU cKO mice, we set up three independent cohorts of RHAU cKO mice with knockout at different ages. The first group of mice was induced at three-week old RHAU cKO mice. All of these mice die with reduced bodyweight within two weeks after the first injection (Figure 3a). After the first injection, the disease starts within five days on average, which is characterized by stopping bodyweight increasing and appearing weakness (Figure 3b).

For the second cohort of RHAU cKO mice, knockout was induced at ten-week old. Interestingly, based on the disease onset and progression pattern, these mice could be equally divided into two groups (Figure 3a). The first group dies within one month after first tamoxifen injection, while the second group dies much later with developed severe paralytic phenotype. Then we mainly focussed on analyzing the second group of these cohort RHAU cKO mice because they developed the progressive paralytic disorder. On average, these mice start showing paralytic phenotype at one month after the first tamoxifen injection. At the same time, the average bodyweight of these mice starts to change (Figure 3c), specifically, the bodyweight starts to increase, and then drops compared with the wild type mice. To ask the question that if the paralytic phenotype is associated with the limb force output defect, these mice were subjected to muscle grip test. Indeed, we found that the limb force is significantly reduced to almost 50% of the wild type control mice (Figure 3e), indicating that RHAU loss of function in these mice lead to muscle dysfunction.

For the third cohort of RHAU cKO mice, RHAU knockout was induced at 27-week old mice in order to look at what the phenotype would be when RHAU loss of function occurs at old stage. Again, we observed that the mice become paralyzed, but the symptom developed faster than the mice that knockout induced at 10 weeks after the first injection. This is further supported by the fact that the bodyweight already starts to change at three weeks after first tamoxifen injection. The bodyweight initially increases, while it decreases at 8 weeks after the first injection, which is much faster than the RHAU cKO mice induced at 10 weeks after the first injection, because the latter drops the bodyweight only at 11 weeks after the first tamoxifen injection (Figure 3d).
To exclude the possibility that the distinct effects observed upon RHAU knockout at different ages are due to different knockout efficiencies, we further analyzed the RHAU knockout efficiency at both DNA recombination and protein level. For tamoxifen induced Cre/loxP recombination, we found that the recombination in one week, after 3-week old induced RHAU cKO mice, level is very similar with the recombination in six weeks after 5-month old induced RHAU cKO mice (Figure 4a). In order to look at RHAU protein level affected by RHAU cKO, a RHAU monoclonal antibody against RHAU C-terminal from 991 to 1007 was used. RHAU knockout efficiency in muscles from 5-month old induced mice is also very similar with the muscles from 3-week old induced mice (Figure 4b). However, we observed that the RHAU protein level in spinal cord from 5-month old induced mice is much lower than RHAU protein in spinal cord from 3-week old induced mice (Figure 4c). Thus, these results indicate that the knockout efficiency in muscle between mice induced at 3-week and at 5-months is similar, while the knockout efficiency for spinal cord from 5-month old induced is even higher than spinal cord from 3-week old induced mice, suggesting that tamoxifen induced knockout efficiency is independent of the age when animal receive knockout induction.

Thus, our comprehensive study on three independent cohorts of RHAU cKO induced at different ages not only demonstrates that RHAU loss of function could lead to progressive paralytic disorder, but also indicates that the onset and progression of paralytic disorder are determined by the age when RHAU is ablated.
Figure 2 Characterization of RHAU conditional knockout mice. a) one week after RHAU knockout induced at three-week old mice b) one month after RHAU knockout induced at 5-month old mice c) six weeks after RHAU knockout induced at 5-month old mice d) Tricep surae muscle from both wild type and paralyzed RHAU knockout mice
a)

Survival Curve

Days after first injection

3-week-old iKO (n=15)
3-week-old WT (n=16)
10-week-old iKO (n=14)
10-week-old WT (n=14)
27-week-old iKO (n=15)
27-week-old WT (n=18)

b)

3-week-old

Bodyweight (g)

Days after first injection

WT (n=5)
 iKO (n=5)
Figure 3: Monitoring lifespan and bodyweight of RHAU conditional knockout mice a) three independent cohorts of RHAU cKO mice induced at 3-week (red), 10-week (blue) and 27-week old (green), respectively. The survival rate was monitored after RHAU knockout. b-d) the bodyweight was monitored for 3-week, 10-week, and 27-week old mice after tamoxifen injection. e) 10-week old induced RHAU cKO mice (blue curve) were tested for grip force at 12 weeks after first tamoxifen injection. Black dot represents WT mice, white dot represents RHAU cKO mice. Both mice were tested every week for 3 weeks (X data). Y-axis indicates the muscle force. All data were expressed as mean ± SEM.

**Inducible paralytic disorder is independent of motor axon degeneration**

Since the RHAU cKO induced at 10-week and 27-week old mice display the progressive paralytic phenotype, we ask the question of whether the paralytic disorder is due to presynaptic or postsynaptic NMJ. To address this question, we looked at the presynaptic nerve by breeding the RHAU cKO mice with Thy1-
mGFP mice, in which almost all the motor nerves are labeled with mGFP. The RHAU cKO mice harboring thy1-mGFP were injected with tamoxifen at both 3-week and 10-week old age in order to induce neuromuscular-paralytic phenotype.

The 3-week induced RHAU cKO mice were sacrificed when they appear weak. Tricep surae muscle muscle was dissected and stained with Alexa 555 conjugated alpha-BTX in order to look at NMJ synapse. To our surprise, the postsynaptic sites visualized by alpha-BTX remain innervated by presynaptic motor axon terminal at tricep surae muscle. The 10-week induced RHAU cKO mice were also examined at the stage when they show paralytic phenotype. Equally surprising, we found that the presynaptic terminal remains innervating postsynaptic site at NMJ, which is similar with the knockout induced at 3-week old RHAU cKO mice. These results strongly suggest that the neuromuscular-paralytic disorder caused by RHAU loss of function is independent of motor axon retraction.

For morphology of both pre- and post-synapse, in the initial experiment with two mice in each WT and cKO group, we frequently observed that there are reduced post-synaptic AChR clusters visualized by Alexa 555 conjugated BTX at NMJ in very weak (3-week old induced) and severely paralyzed (10-week old induced) cKO mice compared with WT mice. However, with two more mice in each group sacrificed at relatively early stage in the paralytic disorder progression, we could not observe striking difference of postsynaptic AChR clusters at NMJ between WT and RHAU cKO mice, except that at some muscle fibers, the NMJ size is reduced with muscle shrinkage in RHAU cKO mice, which makes the NMJ appears immature-like and less perforated shape compared with NMJs in WT mice (Figure 5a). Thus, the previously observed reduction of Postsynaptic AchR clusters was probably due to the shrinkage and degeneration of muscle fibers in RHAU cKO mice.
Figure 4 Validation of RHAU conditional knockout efficiency. a) tamoxifen-induced DNA recombination efficiency in muscle of RHAU cKO mice. b) tamoxifen-induced RHAU knockout efficiency at protein level in muscle western blot. Determined by monoclonal antibody against RHAU C-terminal from 991 to 1007. c) tamoxifen-induced RHAU knockout efficiency at protein level in spinal cord. Immunohistochemistry.
Motoneuron-specific knockout of RHAU does not cause neuromuscular-paralytic disorder

Due to the observation that presynaptic motor axon still innervates postsynaptic site at NMJ in paralyzed RHAU cKO mice, this suggests that the paralytic phenotype in mice is not because of motor axon degeneration. However, we still could not exclude the possibility that the RHAU loss of function in motoneuron does not contribute to the paralysis in RHAU cKO mice. Thus, one way to tease motoneuron and muscle apart is to knockout RHAU in both tissues separately in order to figure out the role of presynaptic site and postsynaptic site in contributing to paralysis in RHAU cKO mice.

In order to knockout RHAU in motor neuron, the RHAU floxed mice were bred with Hb-9 cre mice, in which Cre recombinase is under control by motor neuron specific Hb-9 promoter (Figure 5a, b). Interestingly, motoneuron specific RHAU knockout mice could be born and survive as normal as wild type mice. The motor behavior like walking and grasping is also as normal as wild type mice. There is no obviously abnormal phenotype observed. Thus, these observations indicate that knockout of RHAU in motoneuron is not sufficient to cause paralytic phenotype in mice.

To further look at the presynaptic motor nerve at NMJ in motoneuron-specific RHAU knockout mice, we then bred the mice with thy1-mGFP mice in order to visualize the motor axons at NMJ. Again, we did not observe any abnormal presynaptic terminal and postsynaptic AChR cluster at NMJ in motoneuron-
specific RHAU knockout mice (Figure 5e). Thus, knockout of RHAU protein in motoneuron does not cause significant morphological change at NMJ in mice.
Figure 5: Motor neuron-specific RHAU knockout a) the strategy of knocking out RHAU specifically in motoneuron. b) Breeding strategy to generate motoneuron-specific RHAU knockout mice with mGFP labeled motor axon. c) Conformation of RHAU knockout by PCR d) conformation of RHAU knockout specifically occurred in spinal cord rather than muscle. e) Muscle sections from motoneuron-specific RHAU knockout mice with mGFP labeled motor axon were stained with alpha-BTX. Scale bar 40um.

**Muscle-specific RHAU knockout leads to destabilization of AChR cluster at NMJ**

In order to knockout RHAU protein specifically in muscle, the Cre recombinase under Cytomegalovirus (CMV) promoter was delivered by using Adeno associated virus (AAV). In this AAV, IRES is followed by cDNA encoding GFP, which allows the expression of both Cre recombinase and GFP at the same time.

By injecting this AAV virus into lateral gastrocnemius (LGC) in triceps surae muscle of RHAU<sup>fl/fl</sup>, we could knockout RHAU protein, at the same time, identified with GFP. Three weeks after the AAV injection, the infected muscles are dissected, and both genomic DNA and total protein were extracted to confirm the knockout efficiency. Indeed, we did observe that there is genomic DNA recombination and reduced protein level of RHAU (Figure 5a). The infected muscle sections were fixed and stained with Alex 555-alpha-BTX in order to look at postsynaptic AChR cluster at the NMJ. Very interestingly, we found that the normally continuous AChR clusters were broken down and fragmented into discrete boutons at the NMJ in RHAU knockout muscle fibers; p<0.01 n=3 (Figure 5b, 5d). Moreover, some individual boutons at NMJ became less dense and thinner. At some NMJs in the RHAU knockout muscle fiber, the pretzel-like shape of AChR cluster became disrupted, and the individual fragmented boutons were distributed along the muscle fiber rather than clustered in the middle of muscle fiber (Figure 5c). This observation demonstrates that stability of AChR cluster at NMJ is disrupted upon RHAU knockout, and strongly suggests the muscle intrinsic role of RHAU in maintaining postsynaptic stability at NMJ.
Figure 6: Muscle-specific RHAU knockout. a) Three mice injected with AAV-CMV-Cre were checked for RHAU knockout efficiency three weeks after infection both at genomic DNA recombination (upper panel) and protein level (lower panel) b) RHAU Knockout LGC muscle section was stained with alpha-BTX (red) to visualize AChR cluster at NMJ. overview scale bar 100um c) RHAU Knockout LGC muscle section was stained with alpha-BTX (red) to visualize AChR cluster at
NMJ. Zoomed in scale bar 50um. d) Quantification of destabilized AChR clusters in muscle with RHAU knockout, data are expressed as mean ± SEM. n=3 p<0.01.

The effect of RHAU loss of function on gene expression at NMJ and non-NMJ regions of muscle fiber

Our finding strongly suggests that the deletion of RHAU leads to dysfunction of postsynaptic side at NMJ, which eventually contributes to a neuromuscular-paralytic phenotype in mice. However, the mechanism underlying this phenotype is unknown. To understand how RHAU loss of function causes this NMJ defect, we took advantage of the DNA microarray method to unveil what occurs at the transcriptional level upon RHAU deletion, which may provide insight to the underlying mechanism.

It is well known that the nuclei in muscle fibers can be divided into two classes based on the distance to the neuromuscular synapse. The nuclei underneath NMJ are specialized to transcribe the mRNAs specific for synaptic function. Thus it is important to analyze the effect of RHAU knockout on transcription in synaptic nuclei and non-synaptic nuclei, separately(Kishi et al., 2005). By using laser dissection microscope and mGFP labeled motor nerve terminal, we could isolate the synaptic enriched and non-synaptic enriched region of muscle fiber from the WT and RHAU cKO mice to do gene expression profile analysis, respectively (Figure 7a).

We found 684 genes differentially expressed between synaptic nuclei and non-synaptic nuclei from WT and RHAU KO mice based on ANOVA test (p<0.01) (Figure 7b).
Figure 7: Laser dissection and Microarray. a) Fresh muscle section from RHAU cKO mice with mGFP labeled motor nerve terminal. The mGFP labeled NMJs were dissected and collected. b) All the gene expression values were normalized and tested for statistical significance by using ANOVA analysis (p<0.01). The total of 684 genes that differentially expressed were analyzed by hierarchical cluster, and visualized here.

**Genes known to be enriched at NMJ**

There are some genes known for being specifically enriched at the NMJ. Thus, it is useful to check if these genes are differentially expressed between synaptic enriched and non-synaptic enriched region of muscle fiber in our data. Indeed,
the genes including AChR subunits, adhesion molecules NCAM are specifically highly expressed at the NMJ region compared with the non-NMJ region of muscle fiber, indicating that our expression profile experiment could successfully identify some genes specifically enriched at NMJ. This suggests that our LDM based microarray technique is sound. (Figure 8)

Figure 8: Genes that are known enriched at NMJ were found indeed to be specifically with high expression level at NMJ.

**Genes involved in RNA processing and protein synthesis.**

Based on the cluster analysis, we found that a cluster of genes had lower expression, specifically in the NMJ enriched region from RHAU KO mice compared with other groups. Functional analysis of this cluster, we found that it contained 26 genes encoding ribosomal proteins involved in protein synthesis. Since local protein synthesis plays a very important role in maintaining
functional synapses, this result suggests that local protein synthesis is reduced by RHAU loss of function at NMJ.

We also found that expression level of several RNA binding proteins is affected in RHAU cKO mice. Staufen-2, which is a mammalian homolog of drosophila staufen, is up-regulated in RHAU cKO mice at both NMJ and non-NMJ region of muscle fiber. Staufen-2 is a RNA binding protein expressed in the muscle and specifically enriched at NMJ. In hippocampal neuron, staufen binds to RNAs to transport them along microtubule to the dendritic spine where local protein synthesis occurs (Kohrmann et al., 1999b). Thus, it is possible that staufen performs the similar function at NMJ. The other RNA binding protein called pumilio-2 is also up-regulated upon RHAU KO at both non-NMJ and NMJ region. In Drosophila, pumilio-2 has been shown to be able to function as a translational repressor to regulate the development and function of NMJ by binding to specific mRNA.

Some other genes involved in RNA processing are also identified in this study. The RNase Drosha, involved in microRNA processing (Lee et al., 2003), is found specifically down-regulated at NMJ from RHAU cKO mice. The nuclear transport factor 2 is down-regulated at both non-NMJ and NMJ from RHAU cKO mice compared with WT mice. We also identified several RNA polymerases are down-regulated upon RHAU deletion at NMJ compared with WT. It is worthy to note that RNA helicase DHX15, a gene belong to the same gene family with RHAU, is up-regulated in the RHAU cKO mice at both NMJ and non-NMJ region of muscle fiber. Thus our result suggests that the RHAU loss of function may causes the abnormal RNA processing in the muscle in mice.

a)
b)
Figure 9: Genes involved in RNA processing and translation a) Genes encoding ribosomal proteins are reduced at NMJ in RHAU cKO mice. b) Genes involved in RNA processing are affected in RHAU cKO mice.

**Genes involved in the cytoskeleton and its binding proteins**

Interestingly, we have also identified several cytoskeleton genes and cytoskeleton binding protein in our list. The alpha-actin, cardiac (alpha-catinin), which is muscle-specific actin protein, is down-regulated in muscle from RHAU cKO mice. Meanwhile, two actin binding protein Arp2/3 and Myosin X are both down-regulated in the RHAU cKO mice. It is worthy to mention that Myosin X could function as a molecular motor involved in formation of filopodia (Kohrmann et al., 1999a; Bohil and Robertson..., 2006; Bohil et al., 2006). In addition, a gene encoding dynein heavy chain, which also could function as a molecular motor along cytoskeleton, is down-regulated in muscle of RHAU cKO mice. These results suggest cytoskeleton and its function in transport may be impaired in RHAU cKO mice.

Figure 10: genes encoding cytoskeleton and its binding proteins are reduced by RHAU cKO at NMJ.
Insulin/IGF-1 pathway attenuated in RHAU cKO mice

The Insulin growth factor-1 (IGF-1) is down-regulated in both non-NMJ and NMJ region of RHAU cKO mice. IGF-1 is a very important growth factor for normal function of muscle fiber and NMJ that could also serve as a retrograde signal to motor neuron for its survival. Moreover, two other genes, Insulin receptor substrate 3 (IRS-3) and insulin-like growth factor binding protein 2, are involved in the insulin/IGF-1 signaling, are also down-regulated in the NM region of muscle fiber in RHAU cKO mice (Figure 11). These data suggest that the Insulin/IGF-1 pathway may be attenuated in RHAU cKO mice.

Figure 11: Genes that play roles in Insulin/IGF-1 pathway are affected by RHAU cKO.

Endogenous localization of RHAU protein in vitro and in vivo

Since the RNA binding protein RHAU has been identified as two isoforms existing in vivo, it is important to determine that which isoform RHAU spliced and exists in the muscle fiber and motor neuron. To draw conclusion about this, one way is to use RHAU specific antibody to detect the endogenous expression pattern in the fixed tissue.
First of all, the specificity of RHAU antibody has to be determined. A RHAU polyclonal antibody has been ordered from Proteintech, which was raised from rabbit against the antigen 385 amino acid in the N-terminal of RHAU. The full-length cDNA fused with eGFP was expressed in C2C12 myoblast. After two days when the GFP is visible under microscope, the cells were fixed and stained with RHAU antibody. Consistently, the RHAU protein level identified by RHAU polyclonal antibody is selectively increased in the cells with transfected RHAU-GFP (Figure 12). This indicating the RHAU over-expressed in myoblast could be recognized by this RHAU polyclonal antibody. Moreover, it is obvious that there is co-localization between exogenously expressed RHAU-GFP and the endogenous RHAU protein localized at cytoplasm, which is characterized by color yellow. These results indicates that this polyclonal antibody against RHAU could specifically recognize RHAU protein.

![Image](image-url)

**Figure 12:** RHAU antibody validation. N-terminal GFP fused RHAU full-length cDNA was tranfected in myoblast. After two days, cells were fixed and stained with RHAU antibody (red). Two channels were merged, and the part co-localized is in yellow. Scale bar 50um.

Due to the specificity of this antibody, I then further used this antibody to detect the expression pattern in the C2C12 myotube. Again, we found that the majority of RHAU protein detected is in the cytoplasm (Figure 13). Interestingly, some RHAU proteins are found to localize right along the nuclear envelope, others are
distributed along the myotube in a particle-like or granule-like form (Figure 13), which is very similar to the protein involved in cell trafficking pathway. This prompts us to ask the question that if RHAU is a protein that distributes along the cytoskeleton. We then co-stained RHAU protein with acetylated-alpha tubulin and Alexa 488 fluophore conjuncted phalloidin that specifically binds the F-actin, respectively both in fibroblast and myotube. Surprisingly, we found RHAU specifically co-localized with actin filaments, but not with microtubule (Figure 14). More interestingly, we could not observe that RHAU co-localizing with both cytoskeletons in the myoblast. These results indicate that RHAU is a RNA binding protein associated with actin filament, and suggests that RHAU protein may function as RNA binding protein in a cell trafficking complex to transport RNAs via actin filament.
Figure 13: RHAU distribution pattern in myoblast and myotube. Cultured myoblast and differentiated myotubes are fixed and stained.
Figure 14: RHAU distribution pattern in myoblast and myotube. Cultured myoblast and differentiated myotubes are fixed and stained with RHAU antibody (RHAU), cells are co-stained with acetylated-alpha-tubulin to visualize microtubule.
To ask whether the results observed in myotubes is consistent in vivo, we again used the antibody to check the expression pattern in the fixed muscle fiber in vivo (Figure 15a). RHAU protein co-localizes well with actin filament only on the surface of the muscle fiber but not in the middle of muscle fiber, suggesting that RHAU may perform specialized function for muscle fiber membrane. Interestingly, RHAU is distributed along the surface of muscle fiber but specifically enriched at NMJ, where it does not specifically co-localize with AChR. This raises the question of whether the RHAU protein at the NMJ is localized at the post-synapse, or at pre-synaptic terminal. To answer this question, the sciatic nerve was axotomized in order to remove the presynaptic terminal at NMJ. Five days after axotomy, the tricep surae muscle sections were co-stained with RHAU and Alexa 555 conjuncted alpha-bugarotoxin (Figure 15b). The RHAU protein at NMJ is dramatically reduced in the axotomized mice compared with the NMJ from the mice without axotomy, although there is still some RHAU protein left at NMJ. This result strongly suggests that the RHAU protein enriched at NMJ is mainly from presynaptic terminal.
Figure 15: RHAU distribution pattern in vivo. a) triceps surae muscle section was stained with RHAU antibody (RHAU), Alexa 488-conjugated-Phalloidin (F-actin), 555-
conjungated-BTX. In merged F-actin and RHAU, F-actin is in green while RHAU is in red (single focal plane). In merged RHAU and BTX, RHAU is in green while BTX is in red (MIPs). b) 5 days after sciatic nerve cut, tricep surace muscle sections were stained with RHAU antibody (green) and Alexa-555-BTX (red) (n=2).

Since the RHAU protein exists in the motor neuron terminal, we then further looked at RHAU protein distribution pattern in fixed spinal cord sections. Consistently, we observed RHAU protein localized to the spinal cord ventral root, where the motor axons exit the spinal cord (Figure 16). The distribution of RHAU protein in the ventral root indeed follows the pattern of the motor nerve going out from gray matter to white matter. Furthermore, the RHAU protein is unexpectedly enriched on the membrane of motor neuron with modest expression level in the cytoplasm and nucleus.

![Single focal plane](image1.png) ![MIPs](image2.png)

Figure 16: fixed spinal cord section was stained RHAU antibody. Both single focal plane and MIPs are presented.
Discussion

RHAU belongs to evolutionarily conserved DEAH RNA helicase family, which consists of approximately 15 RNA helicase proteins. The biological function for this gene family remains unknown. Conventional knockout of RHAU leads to embryonic lethality, suggesting the essential role of RHAU in embryonic development. To address the biological function of RHAU in adult animal, conditional RHAU knockout mice were generated in Yoshikuni Nagamine laboratory, loss of RHAU in adult mice develops an age-dependent neuromuscular-paralytic phenotype. This strongly suggests a role of RHAU in maintaining normal neuromuscular function. This thesis is aimed to address the mechanisms underlying how RHAU loss of function leads to age-dependent neuromuscular disorder.

In our study, we could genetically label the motor axons with membrane targeted GFP in order to look at the presynaptic nerve innervation in RHAU cKO mice by breeding with Thy1-mGFP mice generated in the lab before. To our surprise, we find that the postsynaptic AChR clusters remain innervated by presynaptic nerve terminal in paralyzed RHAU cKO mice. This result suggests that the paralytic phenotype in RHAU cKO mice may be not due to presynaptic motor nerve degeneration, but result from postsynaptic side. However, the conclusion could not be drawn unless the role of pre- and post-synapse is clearly teased apart.

We could take the advantage of Cre/loxp system to knockout the RHAU in motor neuron and muscle by using motoneuron-specific Cre line and muscle-specific expression of Cre recombinase, respectively. To our surprise, motoneuron-specific knockout of RHAU could not cause motor axon degeneration, suggesting that presynaptic motor neuron does not play major role in contributing the paralytic phenotype in RHAU cKO mice. By injecting AAV-CMV-Cre to muscle in RHAU fl/fl mice, we could knockout RHAU specifically in the muscle, while leaving motor neuron intact. We found that the postsynaptic AChR clusters start to disassembly at NMJ after muscle-specific RHAU knockout, suggesting that RHAU is involved in maintaining AChR cluster stability. Interestingly, the destabilized postsynaptic AChR clusters found in muscle-specific knockout are different from the AChR clusters observed at NMJ in RHAU cKO mice, in
which the NMJs remain intact. This different morphology observed in global knockout and muscle-specific knockout suggests that the presynaptic signal sent by motor nerve may participate in stabilizing the postsynaptic AChR cluster. If the presynaptic signal exists to stabilize postsynaptic stability, it must be negatively regulated by RNA helicase RHAU, since the AChR clusters remain stable only in the mice with knockout of RHAU in both motor neuron and muscle, while destabilized in the mice with muscle-specific knockout. Thus, it would be very interesting to figure out what the signal sent by motor neuron is. One of the candidates could be neuron-specific form of Agrin, which is a glycoprotein secreted by motor neuron terminal to initiate postsynaptic differentiation.

Our study suggests that the intrinsic signaling in muscle is essential and sufficient for maintaining AChR cluster stability. This cell autonomous signaling phenomenon is also observed during NMJ formation. In both topoisomerase 2β and Hb9 mutant mice (Yang et al., 2000; Yang et al., 2001), the postsynaptic AChR clusters already formed well in the middle of muscle fiber in absence motor nerve innervation, suggesting the muscle-specific signaling is sufficient for initiation of postsynaptic AChR cluster formation. When disrupting muscle-specific kinase MuSK in muscle fiber, the AChR cluster could not form the pre-pattern at NMJ. AChR cluster pre-pattern at NMJ is further refined when motor nerve terminal arrived (Yang et al., 2001). Thus, these results demonstrate that muscle intrinsic signalings play major role in NMJ formation and stability.

Although several factors have been already identified involved in regulating postsynaptic stability, the particular interesting factor is the dystrophin and dystrophin-associated complex (DPC). Why this particular scaffold protein complex is interesting is not only because mutation of many genes in this complex causes neuromuscular diseases such as muscular dystrophy, but also because many genes loss of function in this complex such as dystrobrevin and dystroglycan causes the fragmentation and destabilization of postsynaptic AChR clusters (Grady et al., 1999; Grady et al., 2000), resembling some aspects of NMJ observed in muscle-specific knockout mice. Based on this, it would be interesting to see if there is a linkage between RHAU and the DPC complex. Dystrophin and DPC play structural roles in linking the actin cytoskeleton to
extracellular matrix, stabilizing the sarcolemma during repeated cycles of muscle contraction and relaxation. The DPC also participates in cell signaling through interacting with calmodulin and nNOS (Blake et al., 2002). Thus, once the dystrophin and DPC is disrupted, it would not only impair the muscle membrane integrity, but also cause muscle cell signaling dysfunction, both of which are required for the stability of AChR clusters on muscle fiber membrane. The question raised is that what is the machinery to maintain such a big protein complex stably anchored on muscle fiber membrane. There could be mRNA transport and local protein synthesis happening along the muscle fiber to support the dystrophin and DPC during lifetime in order to maintain the stability of muscle membrane. Since RHAU is an RNA helicase involved in RNA processing, the one possibility is that RHAU regulates transport and translation of mRNAs encoding dystrophin and DPC scaffold proteins in order to maintain post-synaptic AChR cluster stability at NMJ.

Interestingly, the DPC is required for AChR cluster stability but not for formation of AChR cluster (Grady et al., 2000). For RNA helicase RHAU, the issue that if it is essential for formation of AChR cluster at NMJ is unknown. Thus, it would be also interesting to address this question by looking at NMJs from mice with RHAU knockout at early stage of NMJ formation.

RHAU was initially identified as RNA helicase involved in degrading urokinase plasminogen activator mRNA (Tran et al., 2004), suggesting that it may regulate gene expression post-transcriptionally. In order to look at the effect of RHAU loss of function on global gene expression, we did gene expression profile analysis on both NMJ region and non-NMJ region of muscle fiber in RHAU cKO mice. Indeed, there are 684 genes differentially expressed between WT and RHAU cKO mice. One of the notable findings is that some genes encoding ribosomal proteins are specifically down-regulated at NMJs from RHAU cKO mice compared with NMJs from WT mice. This suggests that RHAU may also participate in regulation of translation. We also identified many cytoskeletal genes, further with actin binding molecular motors such as alpha actin cardiac, arp2/3, myosin x, are down-regulated at NMJs from RHAU cKO mice, suggesting that RHAU knockout may affect cytoskeleton stability or function. Interestingly, we found that the expression
levels of several RNA binding proteins such as Staufen-2, and Pumilio-2 are affected by RHAU knockout at NMJ. Both Staufen-2 and Pumilio-2 have been demonstrated before that they are involved in both CNS and NMJ synaptogenesis by regulating specific mRNAs transport and translation at synapses (Khandjian et al., 1996; Kiebler et al., 1999; Kohrmann et al., 1999a; Bélanger and Stocksley, 2003; Menon et al., 2004a; Ye et al, 2004a). This prompts us that RHAU may also participate in mRNA transport and translation at NMJ.

To better understand the cellular function of RHAU, we need to know the endogenous localization of RHAU protein. Fortunately, we have an antibody could specifically recognize the endogenous RHAU protein, providing a chance to look at the distribution pattern of RHAU protein both in vitro and in vivo. Surprisingly, the RHAU protein is mainly found to exist as a particle or granule-like structure mainly localizing in cytoplasm rather than inside the nucleus, suggesting that it may participate in post-transcriptional regulation of gene expression rather than regulating transcription. The distribution of RHAU particles is along nuclear envelope and its co-localization with F-actin in the cytoplasm in myotubes, which is consistent with the idea that RHAU may function as a molecular motor involving in the mRNA transport, since mRNA transcribed in the nucleus has to be exported to cytoplasm, and transported to the destination along cytoskeleton for local protein synthesis. The mRNAs associated with RHAU protein containing particles need to be defined in order to know the specific function of RHAU protein at the NMJ. The co-localization between RHAU and F-actin is also observed in muscle fibers in vivo, indicating that the function of RHAU in muscle fibers may be the same as it is in myotube. Thus, myotubes could be used as a good in vitro model to study many aspects of RHAU protein. It is worthy to note that RHAU protein is enriched at NMJ, specifically, it is enriched at presynaptic terminal at NMJ based on the axotomy study. It is also distributed along motor axons in ventral root in the white matter. This suggests that RHAU protein may play a similar role in mRNA transport and synthesis in motor neuron as in muscle fiber, although presynaptic RHAU is dispensable for maintaining post-synaptic AChR cluster stability at NMJ.
The previous biochemistry study shows that RHAU could regulate gene expression by directly binding and degrading mRNAs (Tran et al., 2004). This suggests that RHAU protein may maintain the post-synaptic stability at NMJ by regulating the turnover of specific mRNAs. However, it is also possible that RHAU regulates gene expression indirectly through its involvement in biogenesis or transporting of small microRNAs, which in turn modulate the synaptic mRNA stability. This has been reported in a recent study in which microRNAs associated with RNA binding protein FMRP could regulate the synaptic structure and function by targeting specific mRNAs (Edbauer et al., 2010). Thus, the specific molecular mechanism underlying that how RHAU loss function causes destabilization of postsynaptic AChR clusters at NMJ and paralytic behavior remains to be elucidated.

In conclusion, the conditional RHAU knockout mice have been comprehensively characterized. The RHAU cKO mice develop age-dependent progressive neuromuscular-paralytic disorder with muscle shrinkage, which is independent of motor axon denervation. Motoneuron-specific RHAU knockout does not show paralytic disorder with normal NMJ morphology. Muscle-specific RHAU knockout leads to destabilization of postsynaptic AChR clusters at NMJ, indicating that RHAU is essential for maintaining postsynaptic stability at NMJ.
Materials and methods

Animal and genotyping

The RHAU exon 8 floxed mice were obtained from Yoshikuni Nagamine lab at FMI. The transgenic mice harboring Cre recombinase fused with Estrogen receptor (CreER) under actin promoter are from Jackson Lab. The line of neuron-specific expressing membrane targeted GFP under Thy1 promoter mice (Thy1-mGFP) breed in our lab as previously described. The motor neuron-specific expressing Cre recombinase mice (Hb9-cre) are from Silvia Arber Lab at Biozentrum, University of Basel. All these mice are maintained 12h/12h light cycle with free access to water and food.

All the mice are genotyped by regular PCR with specific primers. The RHAU exon 8 floxed mice are genotyped by using primer forward TGTACATTTTGATACTACTTAATCTACCCTTTGA and reverse TATGGAAATGCTCCTAGTTAAGTTTAGAGCT at 56 annealing temperature. Both Actin-creER and Hb9-cre transgenic mice are genotyped with same primer for Cre recombinase forward GCAAGAACCTGATGGACATGTTCAG and reverse GCAATTTCGGCTATACGTAACAGGG at 56 annealing temperature.

Immunohistochemistry

Mice are transcardically perfused with iced PBS followed with ice-chilled 4% paraformaldehyde in PBS. The spinal cord and muscle tissue are dissected and left in the perfusion solution for at least two hours. The fixed tissues are left in PBS containing 30% sucrose overnight before sectioning. The tissues are embedded with Tissue tec (Sakura), Sectioning is performed at 50um on a cryostat (micron).

Spinal cord floating sections are used for immunostaining, while immunostaining for muscle sections is performed on glass slides. All sections are washed twice with PBS before blocking and penetration. Washed sections are blocked with fetal bovine serum and horse serum, and then permeablized with triton-x100. Then the sections are washed for primary antibody incubation at 4 degree for two days. Sections are washed with PBS three times before incubating with Alexa fluophore conjugated secondary
antibodies for two hours at room temperature. The sections are washed three times again with PBS and mounted with Prolong mounting media (Invitrogen).

**Western blot**

Fresh tissues dissected from animals are sonicated in the western lysis buffer and centrifuged for 15min at speed of 12000g. Supernatants are transferred into new tubes for further experiments. The protein concentration is determined by using BCA kit (Pierces). The protein samples are boiled with loading buffer at 100 degree for 5min, and then the same amount of protein in each sample is resolved in 8% SDS-PAGE gel before transferring. The separated proteins in SDS-PAGE gel is transferred onto Nitrocellulose membrane (Bio-rad), and then blocked with 5% non-fat milk for 1 hour. The membrane is incubated with primary antibodies in TBST containing 3% BSA overnight, and then washed with TBS tween-100 three times before one-hour HRP conjugated secondary antibody incubation. The membrane is then washed again with TBST for three times. The membrane is then visualized with ECL substrate (Pierce).

**Antibodies**

The monoclonal antibody against the 991-1007 amino acid in the C-terminal of RHAU is obtained from Yoshikuni Nagamine Lab. The polyclonal RHAU antibody raised from rabbit against 385 amino acids in the N-terminal of RHAU is ordered from Proteintech. Alex 555-alpha-bungarotoxin is ordered from Molecular Probe.

**Axotomy and AAV virus injection**

Wild type mice are deeply anesthetized, and then unilateral skin is incised in parallel to the femur. The biceps femoris muscle is cut longitudinally with sharp forceps in order to expose the sciatic nerve. Then, the sciatic nerve is transected completely to prevent the reinnervation. The skin is then stitched back with nylon suture afterwards. Mice with surgery are put back home cages and allowed to recover.

Adeno associated virus (AAV) serotype 2/6 expressing Cre recombinase under CMV promoter is obtained from Vector Biolab in titer of $10^{12}$. The AAV virus is injected into LGC part of tricep surae muscle in RHAU$^{fl/fl}$ mice to induce muscle-specific knockout. For each mouse, $10^{10}$ particles are injected.
For AAV injection, the unilateral skin along edge of tricep surae muscle is incised in order to expose the muscle. A volume of 5ul virus is injected into LGC with Hamilton syringe. After injection, the skin is stitched with nylon suture, and the mice are allowed to recover in the home cage.

**Laser dissection and DNA Microarray**

In order to visualize NMJs without staining alpha-BTX, the actin-CreER\(^{+/−}\); RHAU fl/fl mice are further bred with Thy1-mGFP Line 17 mice to produce actin-CreER\(^{+/−}\); RHAU \(^{+/−}\); thy1-mGFP\(^{+/−}\) mice, which almost all the motor nerve terminals are labeled with mGFP.

3 ten-week old actin-CreER\(^{+/−}\); RHAU \(^{+/−}\); thy1-mGFP\(^{+/−}\) and RHAU \(^{+/−}\); thy1-mGFP\(^{+/−}\) mice are injected with tamoxifen, respectively, in order to induce RHAU knockout. After six weeks, when the KO mice develop mild hindlimb clasp phenotype, all the mice are sacrificed and fresh tricep surae muscle tissues are harvested. The muscle tissues are gradually frozen down in pre-chilled isopentane (Sigma) on dry ice, and store in -80°C before use.

The frozen tissues are moved to -20°C for 2 hours from -80°C, and then embedded and mounted with Tissue-Tek (Sakura) before cryo-cut. Cryo-cut sectioning is performed in RNase-free environment. 10\(\mu\)m sections are cut and further placed on the middle of RNase-free MMI PET-membrane slide (2 sections per slide) for laser dissection. The sections are dehydrated in absolute ethanol for 30 seconds at Room Temperature and left for air dry for 1min before laser dissection.

Laser dissection is performed by using MMI Laser dissection microscope. The GFP-labeled NMJ enriched region and non-NMJ region located at LGC region of tricep surae muscle section are cut with similar size by laser and harvested with MMI isolationcaps, respectively. For each mouse, 40 to 60 cuts in total are collected in tube and added with 50\(\mu\)l lysis buffer for 15min at RT. Samples are transferred to -80°C for further process.

All the subsequent procedures including RNA extraction, labeling, amplification and hybridization are described else and performed by Erik Cabuy in single-cell genomics.
facility at FMI. GeneChip Mouse Genome 430 2.0 Arrays from Affymetrix are used for hybridization and the signals are captured by GeneChip scanner 3000 7G (Affymetrix).

**Myoblast culture and differentiation**

C2C12 myoblast cell line is cultured in growth medium constituting of DMEM (Gibico), 10% fetal bovine serum (Sigma), 1x penicillin-streptomycin and 1xglutamine. C2C12 myoblast transfection is performed by using JetPei (Polyplus transfection) according to the standard protocol.

Myoblast differentiation is stimulated when cells reach 70-80% confluence. The growth medium is then replaced with differentiation medium that constitutes of DMEM (Gibico), 10% horse serum (Sigma), 1x penicillin-streptomycin and 1xglutamine. All the experiments are performed on the myotube after at least 3 days differentiation.

**Statistics**

All data are expressed as mean ± SEM, and plotted by using SigmaPlot software. The student’s T test is used to test the statistical significance between two groups (WT vs. cKO), the ANOVA analysis is used to determine the statistical significance in microarray study. The P value less than 0.05 is considered to be statistical significant.
References


Bélanger G, Stocksley... M (2003) Localization of the RNA-binding proteins Staufen1 and Staufen2 at the mammalian neuromuscular junction. Journal of ....


Kim N, Stiegler A, Cameron T, Hallock... P (2008) Lrp4 is a receptor for Agrin and forms a complex with MuSK. Cell.


Acknowledgement

This thesis is under supervision by Prof. Pico Caroni throughout the whole study process. I would like to thank him for providing me the opportunity to work in this very productive lab, and for his helpful advice and support for this project. I am deeply impressed by his devotion and enthusiasms to science, which set very good example for my future.

I would like to also thank Dr. Yoshikuni Nagamine for generously providing us the RHAU conditional knockout mice, which made our study possible.

I would like to thank to my thesis committee member Prof. Siliva Arber, and Dr. Jan Pielage, who provided very helpful comments and advice for my study, which is essential for this project completion. I would like to special thank to Prof. Silvia Arber for providing HB-9 cre mice.

I would like to thank Dr. Lan Xu and Dr. Smita Saxena for initially teaching me spinal cord and muscle dissection technique, which is essential for this project.

I would like to also thank Dr. Jance Lai for sharing the experience working on RHAU conditional knockout mice, also for participating in generating the Blue survival curve.

I would like to thank all the members of Caroni lab, who provided a very exciting scientific atmosphere, technical help and great comments on this project, which is also essential for this project completion. Special thanks to Mike for him teaching me for Myotube culture and for carefully proofreading.

Finally, I would like to thank all the facilities and staff members at FMI for support, which made FMI as a great place to work.
Curriculum Vitae

Pu Wu

Born on February 16, 1983

Nationality: China

Work address:
Friedrich Miescher Institute for Biomedical Research
Maulbeerstrasse 66
4058 Basel Switzerland
tel: +41 616 97 66 99
fax: +41 616 97 39 76
E-mail address: pu.wu@fmi.ch

Education and Research Experience:

10/2007 – present  Friedrich Miescher Institute for Biomedical Research/ Unibas
Characterization of RHAU conditional knockout mice and the role of RHAU in maintaining post-synaptic AChR cluster stability

09/2004 – 07/2007 Shanghai Institute of Brain Functional Genomics Master of Science
Studying the effect of calorie restriction on neurodegenerative phenotypes in Presenilin-1 and Presenilin-2 forebrain-specific knockout mice.
The hypothalamic gene expression response to calorie restriction during aging.

09/2000 – 07/2004 East China Normal University Bachelor of Science
Selected Publications

Systematic gene expression profile of hypothalamus in calorie-restricted mice implicates the involvement of mTOR signaling in neuroprotective activity.


Calorie restriction ameliorates neurodegenerative phenotypes in forebrain-specific presenilin-1 and presenilin-2 double knockout mice.


Definition of Genes and Paths Involved in Alzheimer's Disease: Using Gene Expression Profiles and Chemical Genetics at the Mouse Brain Level