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Deciphering ColQ induced mechanisms in the control of AChR mRNA levels

Jennifer Karmouch

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THÈSE DE DOCTORAT DE L'UNIVERSITÉ PARIS DESCARTES

Spécialité Génomes, épigénomes, et destin cellulaire

Ecole doctorale

Biochimie, Biothérapies, Biologie Moléculaire et Infectiologie-B3MI

Présentée par

Jennifer KARMOUCH

Pour obtenir le grade de

DOCTEUR de l'UNIVERSITE PARIS DESCARTES

Sujet de la thèse

DECIPHERING ColQ INDUCED MECHANISMS IN THE CONTROL OF AChR mRNA LEVELS

Soutenance le 9 Avril 2014

Devant le jury composé de :

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Jennifer Karmouch

TABLE OF CONTENTS

ACKNOWLEDGMENTS.....	i
TABLE OF CONTENTS.....	iii
LIST OF FIGURES.....	vii
LIST OF ABBREVIATIONS.....	ix
INTRODUCTION.....	1
I. THE CELLULAR COMPONENTS OF THE NMJ AND THEIR ORIGIN.....	4
A. <i>The Birth of the Motor Neuron and its Specification</i>	4
B. <i>Schwann Cell Development</i>	8
C. <i>Skeletal Muscle Development</i>	11
1. Somitogenesis and early myogenesis.....	11
2. Myofiber formation.....	15
II. THE FORMATION OF THE NMJ.....	17
A. <i>Making Synaptic Contact</i>	17
B. <i>Early Events Following Synaptic Contact</i>	18
1. Presynaptic differentiation.....	18
2. Post-synaptic differentiation.....	19
a. AChR structure.....	23
b. Biosynthetic and secretory pathways of AChR.....	25
c. MuSK regulates AChR levels at the NMJ.....	28
i. MuSK structure and activity.....	28
ii. AChR clustering via MuSK and rapsyn.....	29
d. The control of AChR gene expression.....	31
e. Pathologies linked to AChR deficiency.....	34
III. THE MATURATION AND MAINTENANCE OF THE NMJ.....	36
A. <i>Presynaptic Maturation</i>	36
1. Synapse elimination.....	36
2. Interplay between the motor neuron and the Schwann cells.....	38
3. Pathway regulating presynaptic maturation.....	38
B. <i>Post-Synaptic Maturation</i>	39
1. Transformation of AChR clusters during NMJ maturation.....	39
2. The formation of junctional folds.....	40
3. The organization of the synaptic cleft.....	41
a. Laminins.....	41
b. Collagens.....	42
c. Perlecan.....	42
C. <i>NMJ Function: From an Action Potential to a Muscle Contraction</i>	46
IV. COLQ AND MYASTHENIC SYNDROME.....	51

A. <i>ColQ Structure and Function</i>	51
1. The origin of ColQ.....	51
2. The structure of ColQ.....	52
3. Consequences of ColQ deficiency for muscle activity.....	53
a. ColQ and congenital myasthenic syndromes.....	53
b. A mouse model of congenital myasthenic syndrome with AChE deficiency.....	55
4. The roles of ColQ.....	55
a. ColQ and postsynaptic differentiation.....	55
b. ColQ regulates AChR expression.....	56
V. HUR, A UBIQUITOUS POST-TRANSCRIPTIONAL REGULATOR.....	58
A. <i>Post-transcriptional regulation in muscle</i>	58
1. RNA binding proteins.....	58
a. HuR.....	58
b. KSRP.....	59
c. Staufen.....	60
d. CUGBP1.....	61
e. Lin-28.....	62
f. TTP.....	62
B. <i>Hu/Elav Family</i>	62
C. <i>HuR Function</i>	63
1. mRNA degradation pathways.....	63
2. HuR Binding to ARE cis-elements.....	64
D. <i>HuR Expression</i>	65
1. Transcriptional regulation of HuR.....	65
2. The post-transcriptional regulation of HuR.....	65
a. Autoregulation.....	65
b. MicroRNA.....	65
3. Post-translational modifications of HuR regulate its localization.....	66
a. HuR shuttling.....	66
c. Post-translational modifications regulate HuR localization and function.....	66
E. <i>HuR at the NMJ</i>	70
1. HuR stabilises key players of the NMJ.....	70
2. HuR is a therapeutic target of NMJ diseases.....	70
VI. OBJECTIVES OF THE THESIS – DECIPHERING ColQ INDUCED MECHANISMS IN THE CONTROL OF AChR mRNA LEVELS.....	72
A. <i>Identifying ColQ's Phenotypic and Molecular Effects in a Model of CMS with AChE Deficiency</i>	72
B. <i>Deciphering the Mechanism by which ColQ Regulates AChR Subunit mRNA at NMJ</i>	73
C. <i>Providing an Updated and Integrative View of ColQ's Functions</i>	74
RESULTS	75

I.	DECIPHERING THE MECHANISM BY WHICH ColQ REGULATES AChR SUBUNIT mRNA AT NMJ.....	76
	<i>A. Introduction</i>	76
	<i>B. Primary Results</i>	78
	<i>C. Conclusions and Perspectives</i>	80
	<i>D. Article 1</i>	109
II.	PROVIDING AN UPDATED AND INTEGRATIVE VIEW OF ColQ'S FUNCTIONS.....	138
	<i>A. Introduction</i>	138
	<i>B. Article 2</i>	143
	GENERAL DISCUSSION.....	144
I.	FURTHER DECIPHERING THE CASCADE LINKING ColQ TO AChR mRNA LEVELS.....	147
	<i>A. How is ColQ regulating p38 phosphorylation?</i>	147
	<i>B. Activation of p38, a stress response?</i>	147
	<i>C. What is upstream of p38 activation?</i>	148
	1. The MuSK signaling platform.....	148
	2. The disruption of the ECM	149
	i. MuSK Acts as an "ECM sensor".....	149
	ii. Partial denervation.....	150
II.	THE MOLECULAR SIGNATURE OF CMS WITH AChE DEFICIENCY: SPECIFIC AND COMMON TRAITS WITH OTHER CMS, SIGNIFICANCE FOR THERAPEUTIC APPROACH.....	152
	<i>A. ECM gene mutations</i>	152
	<i>B. Therapeutic strategies</i>	152
	REFERENCES.....	154
	ANNEX A: IDENTIFYING ColQ'S PHENOTYPIC AND MOLECULAR EFFECTS IN A MODEL OF CMS WITH AChE DEFICIENCY....	169
	<i>A. Introduction</i>	170
	<i>B. Primary Results</i>	171
	<i>C. Conclusions and Perspectives</i>	174
	<i>D. Article 3</i>	175
	ANNEX B: LIST OF PUBLICATIONS AND COMMUNICATIONS.....	225

LIST OF FIGURES

Introduction

Figure 1. The neuromuscular junction. (p3)

Figure 2. Specification of motor neurons in the vertebrate spinal cord. (p6)

Figure 3. Neural crest cell destiny. (p10)

Figure 4. Myogenesis. (p14)

Figure 5. Hierarchy of transcription factors regulating progression through the myogenic lineage. (p16)

Figure 6. The organization of the maturing post-synaptic membrane. (p21)

Figure 7. The structure of AChR. (p24)

Figure 8. Dynamics of nicotinic acetylcholine receptors at the neuromuscular junction. (p26)

Figure 9. The working hypothesis ACh activates Cdk5 in a manner dependent on calpain. (p30)

Figure 10. Model for stabilization of synaptic gene expression through stabilization of MuSK expression by agrin from motor nerve terminal. (p33)

Figure 11. Synapse elimination. (p37)

Figure 12. The postnatal maturation of the mouse NMJ. (p44)

Figure 13. Schematic of the SNARE/SM protein cycle mediating fusion and the role of synaptotagmin and complexin in Ca^{2+} triggering of fusion. (p48)

Figure 14. Schematic representation of ColQ and its gene mutations leading to CMS with AChE deficiency. (p57)

Figure 15. Regulation of HuR expression localization and function. (p68)

Results

Figure 16. The transcriptional activity of the AChR δ subunit in WT and ColQ^{-/-} myotubes at T2. (p83)

Figure 17. HuR binds endogenous AChR subunit mRNA in myotubes. (p88)

Figure 18. HuR is capable of binding endogenous AChR subunit mRNA in C2C12 myotubes. (p90)

Figure 19. HuR interacts with the conserved ARE motif in the AChR γ 3'UTR. (p93)

Figure 20. HuR interacts with the AChR α 3'UTR. (p94)

Figure 21. The AChR 3'-UTR and its expression of a reporter construct in human embryonic kidney cells. (p97)

Figure 22. HuR localization in WT (MLCL +/+) and ColQ-deficient muscle cells (MLCL -/-). (p100)

Figure 23. The inhibition of JNK does not alter HuR in the absence of ColQ. (p102)

Figure 24. The inhibition of ERK1/2 prevents the upregulation of HuR in the absence of ColQ. (p104)

Figure 25. ColQ regulates AChR mRNA levels at adult NMJs. (p106)

Figure 26. The absence of ColQ does not alter HuR protein levels *in vivo*. (p108)

Figure 27. Synaptic phenotype of ColQ mutant diaphragm at E14. (p141)

Figure 28. Proposed model for the ColQ dependent regulation of AChR subunit mRNA. (p146)

LIST OF ABBREVIATIONS

A	ACh :	Acetylcholine
	AChR:	Acetylcholine receptor
	ARE:	AU rich element
	AUF1:	AU-rich binding factor
B	BD:	Boundary Cap
	BChE:	Butyrylcholinesterase
	bHLH:	Basic Helix Loop Helix
	BMP:	Bone Morphogenetic Protein
C	CARM1:	Coactivator-associated Arginine Methyltransferase 1
	CAST:	CAZ-associated Structural Protein
	Cdc:	Cell division control protein
	Cdk:	Cyclin-dependent kinase
	ChK:	Checkpoint kinase
	CMAP:	Compound Muscle Action Potential
	CMS:	Congenital Myasthenic Syndrome
	CNS:	Central Nervous System
	ColQ:	Collagen Q
	CRM1:	Chromosome Region Maintenance 1
	CT:	Column of Terni
CUGBP1:	CUG-Binding Protein 1	
D	DM:	Dermomyotome
	DM1:	Myotonic Dystrophy Type 1
	DMD:	Duchenne Muscular Dystrophy
	DML:	Dorsomedial
	DMPK:	Dystrophia Myotonica Protien Kinase
	Dok7:	Docking protein 7
	DUSP:	Dual Specificity phosphatase
E	Elav:	Embryonic lethal abnormal vision
	ErbB (EGFR):	Epidermal Growth Factor Receptor
	ECM:	Extracelullar Matrix
	ERK:	Extracellular signal-Regulated Kinase
	ETS:	E26 Transformation Specific
F	FGF:	Fibroblast Growth Factor
G	GABP:	GA Binding Protein
	GAPDH:	GlycerAldehyde 3-Phosphate Dehydrogenase

	GFP:	Green Fluorescence Protein
H	HBS:	Heparin-Binding Site
	HNS:	Nucleocytoplasmic Shuttling Domain
	Hox:	Homeobox
	HuR:	Human antigen R
I	IgG:	Immunoglobulin
	IL4:	Interleukin-4
	IMZ:	Involuting Marginal Zone
J	JNK:	c-jun NH ₂ -terminal kinase
K	KSRP:	KH-domain-Splicing Regulatory Protein
L	Lfng:	Lunaticfringe
	Lmc:	Lateral Motor Column
	LRP4:	Liprotien Related Protein 4
M	MAPK:	Mitogen-Activated Protein
	MAP2K:	MAPK Kinase Kinase
	MKK:	MAPK-Activated Protein Kinase
	MKP:	MAPK Phosphatase
	MY:	Myotome
	Myf:	Myogenic Factor
	MyoD:	Myogenic Differentiation antigen
	MRF:	Myogenic Regulatory Factors
	MuSK:	Muscle Specific Kinase
N	nAChR:	nicotinic Acetylcholine Receptor
	NC:	notochord
	NF- κ B:	Nuclear Factor kappa-light-chain-enhancer of activated B cells
	NMJ:	Neuromuscular Junction
	NPC:	Nuclear Pore Complex
	NRG:	Neuregulin
	NT:	Neural Tube
P	Pax:	Paired box
	PH:	Pleckstrin-Homology
	PI3K:	Phosphatidyl Inositol-3 kinase
	PKC:	Protein Kinase C
	PNS:	Peripheral Nervous System
	PRAD:	Proline-Rich Attachment Domain
	PRiMA:	Proline-Rich Membrane Anchor

	PSM	Presomitic mesoderm
	PTB:	Phosphotyrosine Binding Domain
Q		
R	RA:	Retinoic Acid
	RBP:	RNA Binding Protein
	RNA:	Ribonucleic Acid
	RRM:	RNA Recognition Motif
S	SC:	Sclerotome
	SE:	Surface Ectoderm
	Shh:	Sonic hedgehog
	SIRPS:	Signal Regulatory Proteins
	Six:	Sine oculis homeobox
	SMA:	Spinal Muscle Atrophy
	SMN:	Survival Motor Neuron
	SNAP:	Synaptosomal-Associated Protein
	SNARE:	SNAP Receptor
	Src:	Sarcoma, proto-oncogenic tyrosine kinase
	SRY:	Sex determining region Y
	Sauf:	Staufen
T	TGFβ:	Transforming Growth Factor β
	TIA-1:	Intracellular Antigen-1
	TLDA:	Taqman Low Density Array
	Trm:	Trimerization domain
	TRP:	Tetratricopeptide Repeats
	TTP:	TTR-RBP Tristetraprolin
U	UTR:	Untranslated Region
V	VDCC:	Voltage-Dependent Calcium Channel
	VLL:	Ventrolateral
W	Wnt:	Wingless

INTRODUCTION

Six hundred different skeletal muscle types are responsible for the posture, voluntary movement, and locomotion of the human body. The hundreds of fibers within the skeletal muscle are innervated by axons of the motor neuron forming active synapses called the neuromuscular junction (NMJ) at their site of contact. Each motor neuron can innervate thousands of muscle fibers within the same muscle, but only one terminal axonal branch from the motor neuron will innervate a single muscle fiber.

The NMJ is a synapse that activates muscle contraction, a function that relies on cholinergic neurotransmission in mammals, and its integrity is indispensable for survival. Its formation requires the functional interaction of three cell types: a motor neuron, a Schwann cell and a skeletal muscle fiber. Because of its rather simple organization and accessibility, the NMJ is the best-characterized model synapse in terms of physiology and development. The stimulation of the motor neuron evokes the release of Acetylcholine (ACh) into the synaptic cleft where it binds and activates the acetylcholine receptors (AChR) clustered on the post synaptic membrane (Sanes and Lichtman, 2001). The binding of ACh to AChR results in the depolarization of the muscle membrane inducing the opening of Na-voltage dependant channels. Consequently to the activation, an action potential is generated and quickly spreads throughout the muscle membrane, triggering a muscle contraction (figure 1).

The NMJ has contributed greatly to the understanding of the general principles of synaptogenesis and of the pathophysiological mechanisms underlying a number of neuromuscular disorders.

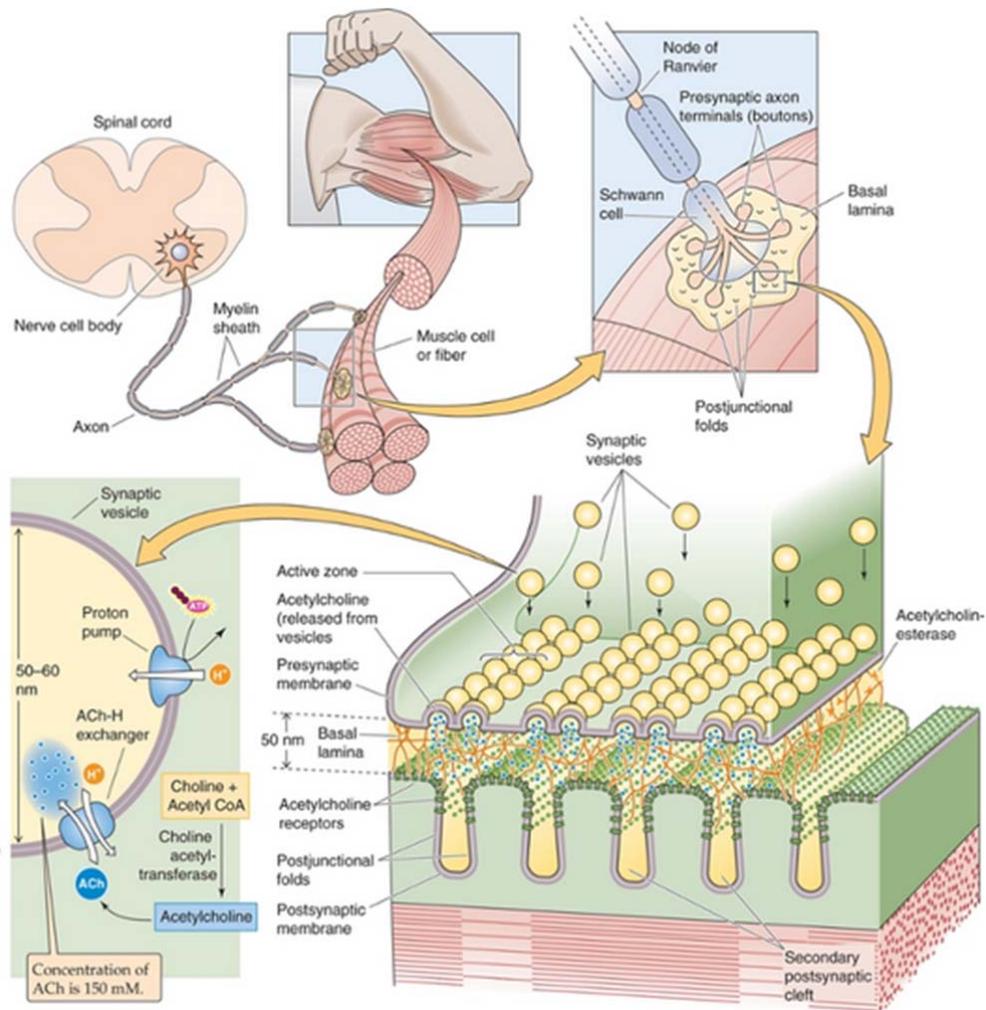


Figure 1. The neuromuscular junction. The motor neuron extends its axon from the motor from the spinal cord where it is guided by its growth cone to the central region of each skeletal muscle fiber within a bundle. The interplay between the Motor axon Schwann cells, and skeletal muscle fibers regulate muscle contractions vital for the posture, voluntary movement and locomotion of the human body (Boron and Boulpaep, 2012).

I. THE CELLULAR COMPONENTS OF THE NMJ AND THEIR ORIGIN

A. The Birth of the Motor Neuron and its Specification

Motor neurons are specific neurons located in the central nervous system (CNS) that control muscle movement. They relay signals from their soma in the spinal cord to the skeletal muscle via their axons, which project outside the CNS. Motor neurons can be classified into three groups: 1) Somatic motor neurons that innervate skeletal muscles, 2) Special visceral motor neurons that innervate bronchial muscles and 3) General visceral motor neurons that indirectly innervate cardiac muscle (Sanes et al., 2011).

Motor neurons are derived from the neuroectoderm of the developing embryo. As a newly fertilized egg develops it endures multiple cleavage and division events to form the blastula which through the process of gastrulation forms the three layers of the embryo: ectoderm, mesoderm, and endoderm. The ectodermal layer gives rise to the neuroectoderm in a process which begins upon the differentiation of a group of cells at the surface of the gastrula. These cells form the neural plate. Involuting cells underneath the neural plate come together to form the notochord. At the same time, the neural plate folds and dissociate from the flanking ectoderm to form the neural tube. The epidermis and notochord send signals to the neural tube which polarise the neural tube by defining the roof plate and floor plate respectively. The rostral region of the neural tube will give rise to the forebrain, mid brain, and hind brain while the remaining caudal part gives rise to the spinal cord (Figures 2A and B).

Motor neurons are derived from the ventral axis of the neural tube. The notochord and floor plate release sonic hedgehog (Shh), a secreted morphogen that establishes a signalling gradient into the ventral neural tube. The roof plate releases Bone Morphogenic Protein (BMP) to counteract the Shh gradient (Figures 2A and B). Together, the BMP morphogens, Shh and retinoic acid (RA; expressed by the paraxial mesoderm) form opposing gradients

that initiate a complex pattern of transcription factors, defining the fate of cellular regions. As a result, the ventral progenitor cells destined for a motor neuron fate will express Class I homeobox genes, specifically Pax6, Nkx6.1 and Nkx6.2, Shh and RA (Figure 2C).

The activation of the motor neuron differentiation pathway is in part induced by Nkx6.1 through its activation of the basic Helix-Loop-Helix (bHLH) transcription factors OLIG2. OLIG2 targets motor neuron specific transcription factors that initiate spinal progenitor cells down the motor neuron pathway (Figure 2C). Once the motor neurons are formed, Shh and RA gradients will define the general characteristics of motor neurons, such as projections of the axons and the release of acetylcholine (ACh) as the primary neurotransmitter. In the mouse, these generic characteristics are commenced at embryonic day 9.5 (Jessell, 2000; Sanes et al., 2011).

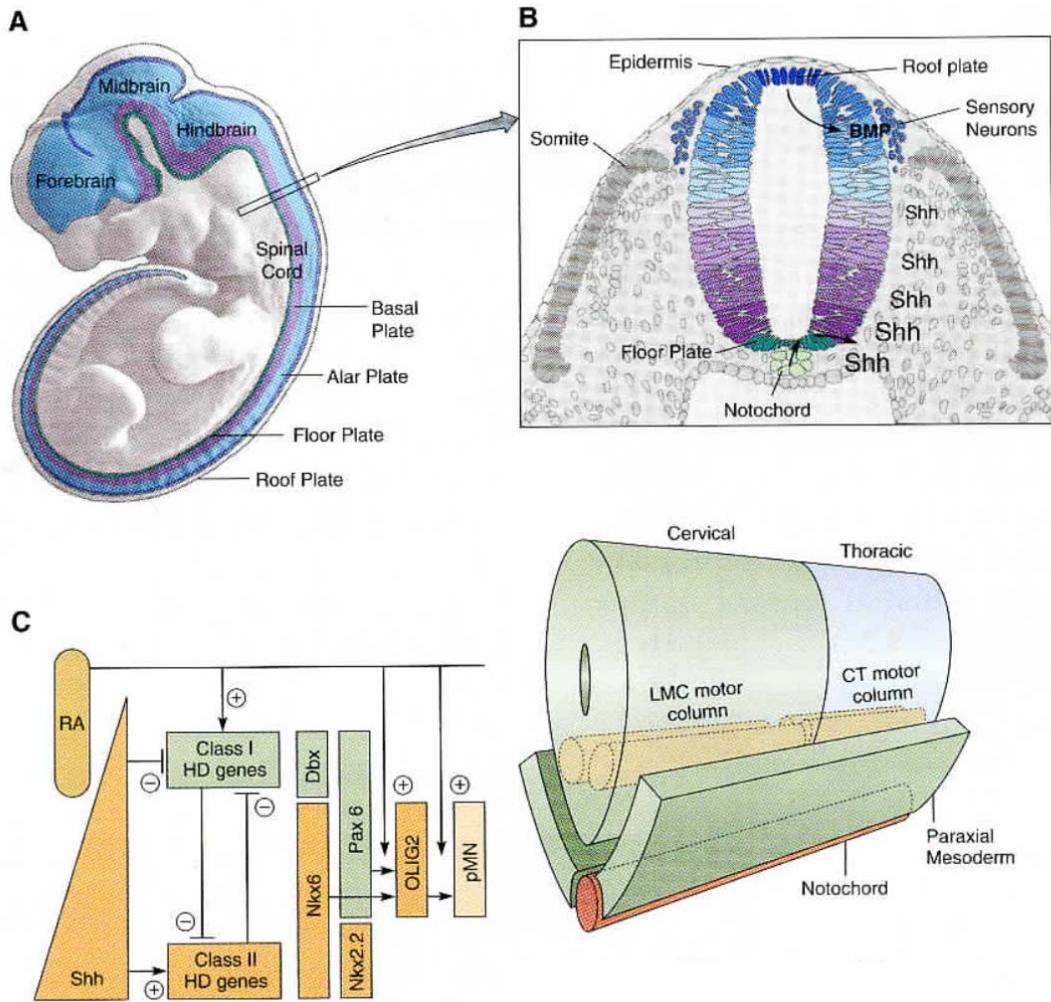


Figure 2. Specification of motor neurons in the vertebrate spinal cord

- A. The neural tube, shown here for a mouse, is subdivided into four longitudinal domains: the floorplate, basal plate, alar plate, and roof plate. Motor neurons are derived from the basal plate.
- B. Schematic cross section of the neural tube. The notochord, which is located underneath the floorplate, releases sonic hedgehog (Shh) which induces the floorplate to release its own Shh. This forms a gradient in the neural tube with high concentrations ventrally and low concentrations dorsally. BMP molecules released from the dorsal epidermis and roof plate form an opposing gradient.

C. (left) A gradient of *Shh* emanates from the notochord and floorplate; threshold levels of *Shh* turn on Class II HD genes. Retinoic acid (RA) expressed by the paraxial mesoderm induces the expression of Class I HD genes that are turned off more ventrally by threshold levels of *Shh*. Class I and Class II HD transcription factors cross-repress each other, creating sharp definitive boundaries at different dorso-ventral levels of the cord. Thus the boundary between *Dbx* and *Nkx6* is more dorsal than the boundary between *Pax6* and *NKx2.2*. Between these boundaries, the *OLIG2* bHLH transcription factor necessary for motor neuron specification is turned on by the concerted action of RA, *Nkx6*, and *Pax6*. *OLIG2* and RA are necessary for the expression of motor neuron differentiation genes of the pMN family. (Below) A gradient of *FGF8* emanates from the mesoderm. High levels of *FGF8* turn on more caudal *HoxC* genes, whereas RA and low levels of *FGF8* turn on rostral *HoxC* genes. Rostral and caudal *HoxC* transcription factors cross-repress each other, creating sharp definitive boundaries at different rostrocaudal levels in the cord. The boundary between *Hoxc6* and *Hoxc9* establishes the boundary between the LMC of the cervical cord and the CT of the thoracic cord (Sanes et al., 2011).

Further maturation of the motor neurons is focused on their diversification into functional groups in the ventral spinal cord. The motor neurons in the spinal cord are organized into two functional columns that project to different muscle groups along the anterior to posterior axis. The first column is called the Lateral Motor Column (LMC) and is located in the cervical region and innervates forelimb muscles. The second is the Column of Terni (CT), located in the mid-thoracic region and innervates the sympathetic chain (Sanes et al., 2011).

The anterior to posterior differentiation of the motor neurons into their specific columns is accomplished by Fibroblast growth factor (FGF8) gradients secreted by the paraxial mesoderm (High FGF8 posterior expression, low FGF8 anterior). This gradient establishes domains of different Hox gene expression, with the posterior domain inhibiting the expression of anterior Hox genes and vice versa. The co-repression of Hox genes creates boundaries for the development of different motor columns of the ventral spinal cord. Motor columns are further subdivided into classes that innervate groups of muscle based on different combinations of the LIM homeodomain (LIM-HD) family of transcription factors. Lastly, these classes are then divided into motor pools that innervate specific muscles. Motor pools are defined by the E26 transformation-specific or E-twenty-six (ETS) family of transcription factors (Sanes et al., 2011).

B. Schwann Cell Development

Schwann cells of the peripheral nervous system (PNS) are important for the survival and maintenance of the motor axon. Throughout their development, Schwann cells aid in such processes as conduction of impulses along axons, nerve development and regeneration, trophic support, participation to the production of the nerve extracellular matrix, and the modulation of neuromuscular synaptic activity.

Schwann cells are derived from the neural crest cells, a transient stem cell population located at the most dorsal axis of the neural tube in the “trunk” region where the neuroectodermal folds fuse to form the neural tube. Once at the trunk, crest cells destined for Schwann cell development migrate along the neural tube ventrally (Figure 3).

Schwann cell development passes through three stages: (1) The formation of neural crest cells present in the immature connective tissue, (2) The generation of Schwann precursor cells associated with axons and (3) Immature Schwann cells which persist till birth. Initially, immature Schwann cells interact with a bundle of axons but quickly initiate radial sorting isolating large diameter axons. Depending on the axon they are associated with, immature Schwann cells will form either non myelinating or myelinating Schwann cells (Jessen and Mirsky, 2005; Sanes et al., 2011; Woodhoo and Sommer, 2008).

In the mouse, these three stages of Schwann cell development occur from embryonic day 10 to the neonatal period. The factors involved in the early stages of Schwann cell maturation are still unclear, however the activation of the transcription factor SOX10 (SRY-related HMG-box 10) is required for the crest cell to glial lineage transition. Axon derived neuregulin 1 (NRG1) and Notch have both been found to promote Schwann cell survival and proliferation and may help accelerate the Schwann cell precursor-Schwann cell transition.

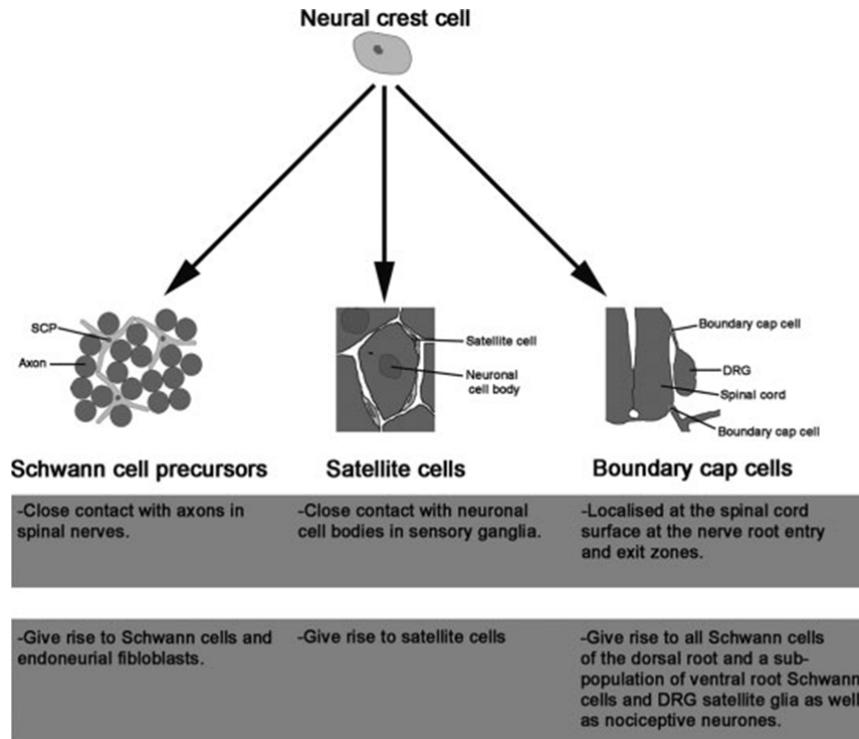


Figure 3. Neural crest cell destiny. Neural crest cells give rise to various types of peripheral glia distinguished by their localization, specific marker expression, factor responsiveness, and function. Schwann cells along peripheral nerves, satellite cells in peripheral ganglia, and boundary cap cell-derived Schwann cells in nerve roots represent distinct peripheral glia subtypes generated from the neural crest (Woodhoo and Sommer, 2008).

C. Skeletal Muscle Development

Skeletal muscle is a dense, fibrous contractile tissue which exists throughout the entire body, and functions to allow body movement by applying force to bones and joints, via contraction. Its formation occurs between embryonic day 7.5 to 14 in the mouse (Buckingham and Rigby, 2014). An overview of skeletal muscle development in the embryo is shown in Figure 4. Figure 5 provides insight into the hierarchy of transcription factors regulating myogenesis.

1. Somitogenesis and early myogenesis

Somites are groups of cells that form sequentially from the paraxial mesoderm also known as the presomitic mesoderm (PSM), flanking the neural tube. Somite formation (Somitogenesis) has been hypothesised to follow a “clock and wavefront” model (Gilbert and Gilbert, 2010). The clock incorporates FGF, Wnt and Notch Pathways, with the FGF and Wnt pathways interacting to activate the Notch pathway. Notch, the transcription factor *Hes7*, a general repressor of the BHLH family, and *Lunaticfringe* (*Lfng*) act as the clock for somitogenesis, through the oscillation in their gene expression (Gilbert and Gilbert, 2010; Saga, 2012). The mechanism involved in the wavefront is still unclear, but it has been proposed to include FGF8, Wnt, and retinoic acid (Gilbert and Gilbert, 2010; Naiche et al., 2011). Both Wnt and FGF maintain the PSM population in an undifferentiated state, by upregulating each other’s expression. Furthermore, FGF has been shown to independently inhibit somitogenesis and thus as the embryo elongates, the PSM are displaced further away from the highly concentrated posterior source of FGF (Naiche et al., 2011). The point of segmentation is defined when FGF signalling is reduced below a threshold and the oscillating clock is in the on state. The boundary between somites results from the intersection of the caudal-rostral gradient, with the rostral-caudal gradient formed by FGF8, Wnt and retinoic acid respectively. Once somites have been cleaved, they immediately undergo epithelialization

(Gilbert and Gilbert, 2010). The newly formed somite is a mesenchymal mass which must be defined into an epithelium with an internal mesenchyme.

Somites are surrounded by the neural tube, notochord, epidermis, and the lateral mesoderm. These surrounding tissues secrete paracrine factors, which depending on their distance, will commit regions of the somite to specific cell types. The ventral medial epithelial cells of the somite closest to the neural tube, which secretes Noggin (Nog) and Shh, form what is called the sclerotome, which contains cells destined for the bones and cartilage. Nog and Shh induce the epithelial cells to be reverted back to mesenchymal cells through the activation of the transcription factor Pax1. Pax1 is also required for the differentiation of cells of the sclerotome into cartilage. Noggin and Shh also induce the expression of Imf (inhibitor of MyoD family), an inhibitor of a group of myogenic transcription factors (Chen et al., 1996; Gilbert and Gilbert, 2010).

The remaining dorsal epithelial cells form the dermomyotome, which having a high expression of Pax3 and Pax7 will go on to form the myotome and the central dermomyotome (Bentzinger et al., 2012). The Myotome is formed from the two lateral “lips” of the dermomyotome. These two lips are the closest and furthest away from the neural tube. Cells of the myotome then migrate underneath the dermomyotome and form a layer of two types of myoblast. The myoblasts furthest away from the neural tube form hypaxial muscles (muscles of limbs and tongue) and the myoblasts closest to the neural tube differentiate into the epaxial muscles (intercostal muscle and vertebrate muscle) (Bentzinger et al., 2012; Gilbert and Gilbert, 2010).

In order for myoblasts to be formed in the somite, Wnt signalling must be present and BMP must be absent (Reshef et al., 1998). Interestingly, Shh and Nog have been shown to inhibit BMP4, ensuring the myoblast state (Marcelle et al., 1997). Thus, the formation of epaxial myoblasts are dependent on the neural tube secretion of high levels of Wnt1 and Wnt3a and

low levels of Shh, while the hypaxial mesoderm is dependent on the secretion of Wnts from the epidermis (Gilbert and Gilbert, 2010).

The myogenic program is induced by the Shh dependent high expression of two bHLH family members, MyoD and Myf5 (Bentzinger et al., 2012). These proteins bind the E-box in the promoter region to control the expression of their target genes. MyoD and Myf5 are part of a group of transcription factors called the Myogenic Regulatory factors (MRF's). MRFs activate the transcriptional cascade needed for the differentiation of skeletal muscle (Bentzinger et al., 2012; Gilbert and Gilbert, 2010). Mrf4 is the third member of the MRF family and has been recently shown to act similar to MyoD and Myf5 as a determining factor for a myogenic cell fate (Kassar-Duchossoy et al., 2004).

The central region of the dermomyotome undergoes an epithelial mesenchymal transition, a process induced by neurotrophin-3 and Wnt1, both of which are secreted from the neural tube. As these newly formed mesenchymal cells undergo mitosis, their daughter cells will either enter into the myotome or relocate dorsally to become part of the dermis (Bentzinger et al., 2012; Gilbert and Gilbert, 2010). Those cells that enter into the myotome will differentiate into myoblast or remain in their undifferentiated state as satellite cells to be used in post-natal muscle growth (Gilbert and Gilbert, 2010).

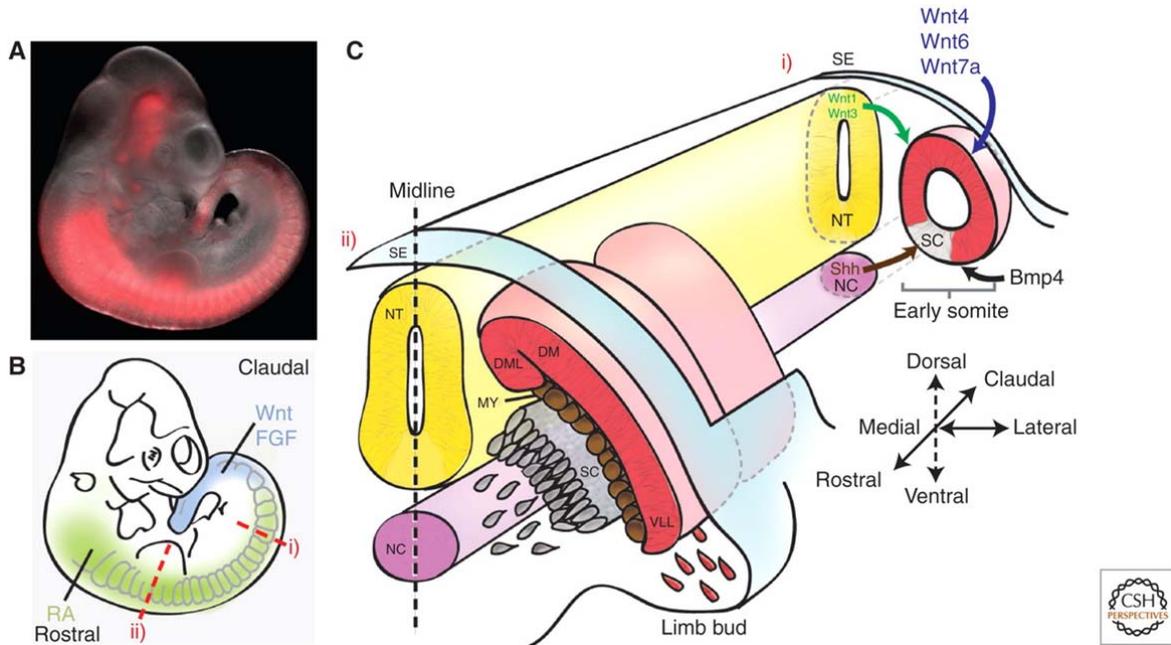


Figure 4. Myogenesis. (A) Embryonic day 10.5 (E10.5) mouse embryo carrying an *Myf5* lineage tracer that induces irreversible expression of a red fluorescent protein. Expression can be observed in the presomitic mesoderm, the somites, and in several head structures. (B) Illustration of the morphogen gradients along the rostral–caudal axis of the embryo. (C) Schematic of transverse sections through the embryo at early (i) and late (ii) stages of somitogenesis. (Ci) Morphogens secreted from various domains in the embryo specify the early somite to form the sclerotome (SC) and dermomyotome (DM). Wnts secreted from the dorsal neural tube (NT) and surface ectoderm (SE) along with bone morphogenetic protein (BMP) from the lateral plate mesoderm maintain the undifferentiated state of the somite, whereas Sonic hedgehog (Shh) signals from the neural tube floor plate and notochord (NC) to induce the formation of the sclerotome. (Cii) As the sclerotome segregates, muscle progenitor cells (MPCs) from the dorsomedial (DML) and ventrolateral (VLL) lips of the dermomyotome mature to give rise to the myotome (MY). At the level of the limb bud, Pax3-dependent migrating MPCs delaminate from the ventrolateral lips to later give rise to limb muscles (Bentzinger et al., 2012).

2. Myofiber formation

Myoblast fusion into myofiber occurs in four main steps (Gilbert and Gilbert, 2010):

- a) **Cell Cycle Exiting** – Proliferating myoblasts are maintained by FGFs and thus its removal induces the myoblasts to secrete fibronectin into its extracellular matrix. Once in the ECM, fibronectin binds the $\alpha5\beta1$ integrin, halts cell proliferation and instructs the myoblasts to differentiate.
- b) **Alignment of Myoblasts** - Cell membrane glycoproteins, such as Cadherins and cell adhesion molecules (CAM), align myoblasts in a chain-like formation. Cytoplasm are also re-arranged by actin to allow for accurate cell contact.
- c) **Cell Fusion** - Myoblast fusion into myofibers is dependent on Calcium ions and is mediated by the metallo proteinases called metrinins. Once myoblasts are induced to fuse, they activate the β HLH transcription factor myogenin, which goes on to activate the expression of muscle specific genes. Myogenin is the last member of the MRF family of transcription factors and is expressed in the myoblast to aid in myoblast fusion and myofiber formation (Bentzinger et al., 2012).
- d) **The “Healing Step”** – Opposing membranes of the fused myoblasts are re-sealed with the help of myoferlin and dysferlin, which have been known to stabilise phospholipids. Newly fused myofibers secrete interleukin-4 (IL4) which recruits other myoblasts to fuse with the tubes. After birth, the expression of the TGF- β protein myostatin inhibits the growth of myofibers and their number.

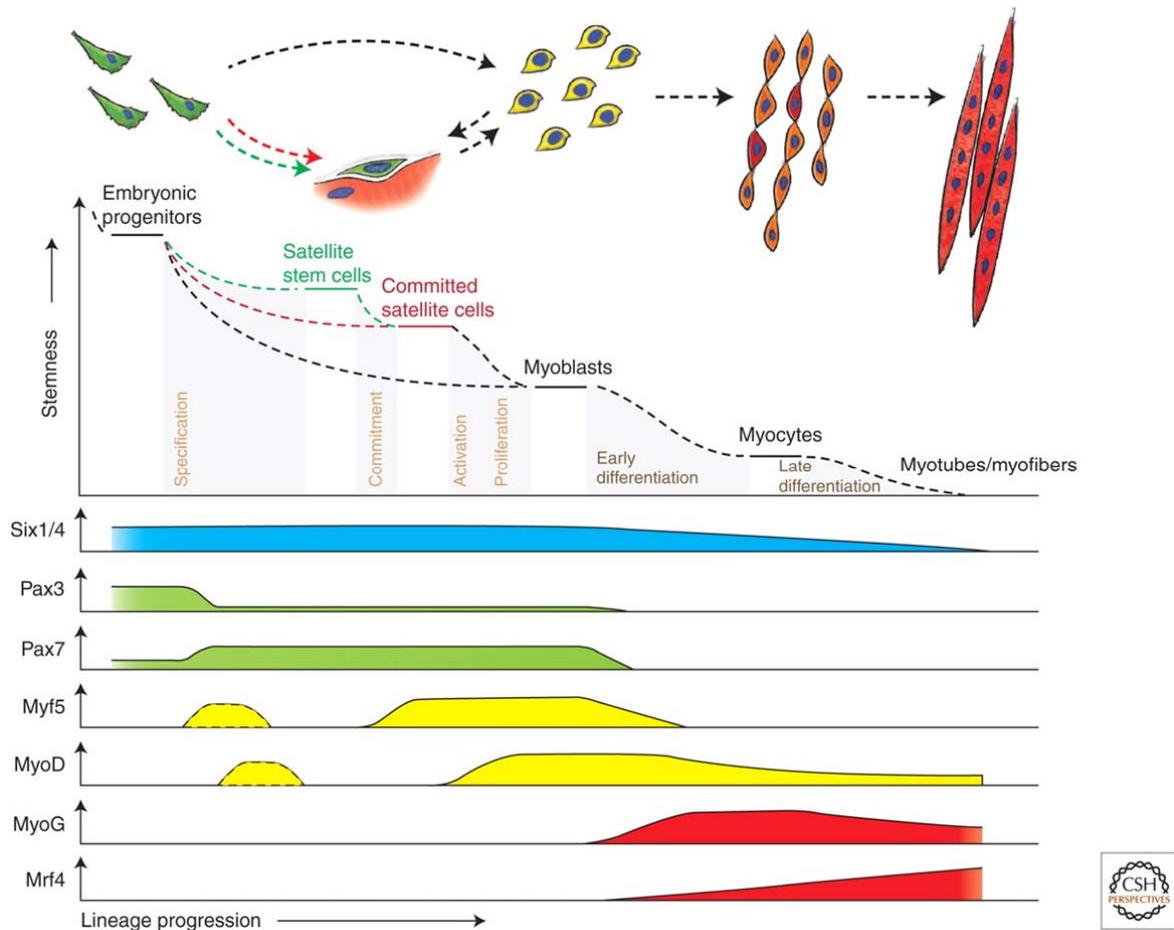


Figure 5. Hierarchy of transcription factors regulating progression through the myogenic lineage.

Muscle progenitors that are involved in embryonic muscle differentiation skip the quiescent satellite cell stage and directly become myoblasts. Some progenitors remain as satellite cells in postnatal muscle and form a heterogeneous population of stem and committed cells. Activated committed satellite cells (Myoblasts) can eventually return to the quiescent state. Six1/4 and Pax3/7 are master regulators of early lineage specification, whereas Myf5 and MyoD commit cells to the myogenic program. Expression of the terminal differentiation genes, required for the fusion of myocytes and the formation of myofibers, are performed by both myogenin (MyoG) and MRF4 (Bentzinger et al., 2012).

II THE FORMATION OF THE NMJ

A. Making Synaptic Contact

The formation of the neuromuscular junction (NMJ) requires the precise opposition of a pre and post synaptic domain through a complex interchange of information between the pre- and postsynaptic partners, including activity dependent and independent processes. In vertebrates, synaptogenesis occurs shortly after myoblast fusion, initiating on embryonic days E13–E14 (Witzemann, 2006). Synaptogenesis follows the extension of the axon from the motor neuron, which is guided by its growth cone, to the central region of the muscle fiber, making initial contact on day E13/14 (Sanes and Lichtman, 2001; Witzemann, 2006).

The extension of the axon of the motor neuron from the neural tube occurs from its ventral exit point and depends on a combination of repulsive and attractive signals from the floor plate and midline. Signals from the surrounding mesenchyme then induce these axons to exit the neuroepithelium. Once at the ventral exit point motor axons are surrounded by a subtype of neural crest cells called boundary cap (BD) cells that ensure axons exiting but prevents the motor neuron cell body from leaving the CNS. Once exited, motor axons grow towards the muscle. The axons then take a ventral, dorsal or axial path depending on the muscle which they will innervate. Once at their respective muscle, individual growth cones are guided by environmental cues, ensuring their positioning at the correct muscle region (Bonanomi and Pfaff, 2010).

It has been postulated that the central domain in the mammalian muscle fiber contains aggregate of macromolecular complexes, including the Acetylcholine Receptors (AChR), that aid in guiding the innervating motor neuron and confirming its synaptic location (Sanes and Lichtman, 1999). This muscle “pre-patterning” is dependent on the post-synaptic protein muscle specific tyrosine kinase (MuSK) and its co-receptor, the low density lipoprotein related protein 4 (LRP4), which forms a complex with MuSK, stimulating MuSK kinase

activity (Lin et al., 2011; Yang et al., 2001). MuSK is expressed primarily in the central region of the muscle prior to innervation and is believed to dictate the accumulation (clustering) of AChR in the middle of the muscle, followed by the terminal phase of axon guidance to the muscle (Kim and Burden, 2008) (Figure 6A).

Once the motor neuron has made contact with the muscle, it releases the neuronal form of the proteoglycan agrin, which binds LRP4, stimulating further association between LRP4 and MuSK, and increases MuSK kinase activity (Kim et al., 2008b; Zhang et al., 2008). This increase in MuSK activity leads to an increase in AChR clustering and density, resulting from a transcriptional and non-transcriptional mechanism. (Gautam et al., 1996; Kishi et al., 2005). It has been hypothesized that LRP4 is capable of acting bi-directionally, stimulating presynaptic differentiation (Yumoto et al., 2012) independently of agrin, through its binding to the motor neuron axons and inducing the clustering of synaptic-vesicles and active zone proteins.

B. Early Events Following Synaptic Contact

1. Presynaptic differentiation

When the motor neuron first makes contact with the muscle fiber, it resembles a bulb-like enlargement. However, as the presynaptic terminal differentiates, the filopodia of the growth cone retract, the synaptic vesicle carrying the neurotransmitter Acetylcholine (ACh) increase and accumulate at the nerve terminal active zone and are subsequently released as the nerve terminal becomes polarized (Sanes and Lichtman, 1999).

Scientists using electron microscopy have identified the active zone as an electron dense thickening of the presynaptic motor terminal, where synaptic vesicles accumulate and dock. The active zone **plays a central role in synaptic transmission** and is formed directly opposing the postsynaptic muscle fiber (Couteaux and Pecot-Dechavassine, 1970; Harlow et

al., 2001; Hirokawa and Heuser, 1982; Nagwaney et al., 2009). The active zone is where synaptic vesicles carrying neurotransmitter ACh are released (Propst and Ko, 1987).

The extracellular matrix (ECM) plays a large role in active zone formation and presynaptic differentiation with collagen $\alpha 1/2$ (IV) chains, directing the initial differentiation of the presynaptic terminal (Fox et al., 2007). Laminin $\beta 2$, an ECM protein secreted by the muscle, has been found to organize active zone formation through its binding to the presynaptic voltage-dependent calcium channels (VDCCs) on one end and Dystrophin on the other. Laminin $\beta 2$ anchors VDCCs and accurately positions them in the active zone allowing for the recruitment of active zone specific proteins including Bassoon and CAST/ERc2/ELKS2 α to VDCCs' intracellular domain (Nishimune, 2012). VDCCs function as scaffolding proteins that link the extracellular proteins to cytosolic active zone proteins (Nishimune, 2012).

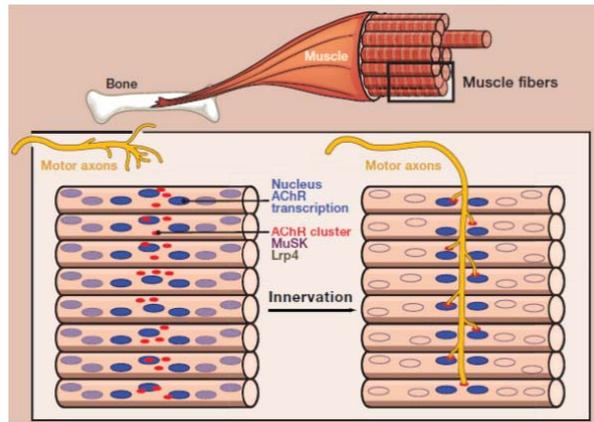
Other factors involved in presynaptic differentiation include the TGF β family of proteins. Mutations in genes that encode TGF β family proteins have been found to disrupt T-bars and presynaptic high density areas where synaptic vesicles accumulate (Wu et al., 2010). Furthermore, Fibroblast Growth Factors (FGFs) and Signal Regulatory Proteins (SIRPS) have been demonstrated to induce synaptic vesicle clustering at the nerve terminal (Fox et al., 2007).

2. Post-synaptic differentiation

A defining feature of post-synaptic differentiation is the re-distribution of muscle AChRs following innervation, with the formation of high-density AChR clusters at the postsynaptic membrane and their dispersion outside the NMJ (Sanes and Lichtman, 2001). Early in vertebrate myogenesis, around embryonic day E13, AChR subunit gene expression and assembly takes place throughout the myofiber. AChR is inserted into the membrane of the myofiber in clusters that are formed and regulated by the transmembrane tyrosine kinase

MuSK (Figure 6B). The importance of the accurate control of AChR expression, insertion and clustering at the post-synaptic membrane is highlighted by the fact that alterations in their membrane density and kinetics are associated with congenital myasthenic syndromes.

A.



B.

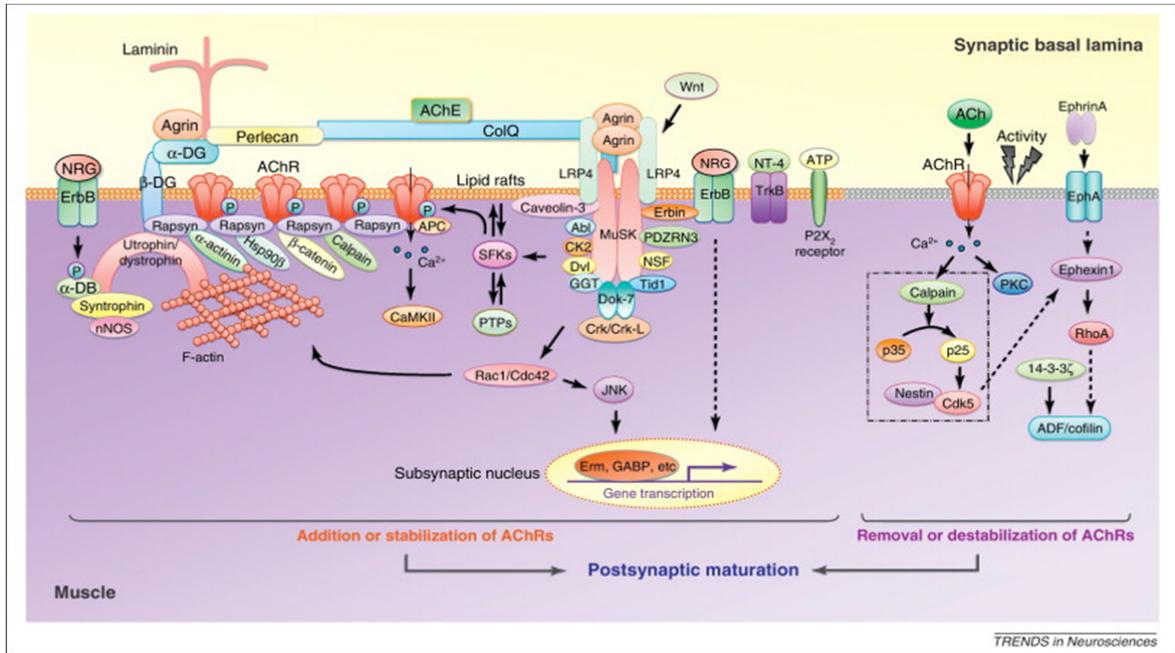


Figure 6. The organization of the maturing post-synaptic membrane

A. The mammalian muscle fiber contains an increase in the expression of groups of macromolecular transmembrane complexes including AChR (blue) along with their clustering in the central region of the muscle fiber (red); a process independent of innervation. The formation of this central domain requires LRP4 and MuSK. The motor axons are thought to approach this region of the muscle, marked by nerve terminals juxtaposed to AChRs. (Adapted from Burden snapshot)

B. Postsynaptic maturation is regulated by positive and negative signaling pathways for AChR stabilization. Positive signaling pathways – including those activated by the MuSK receptor, the dystroglycan–glycoprotein complex, and ErbB and TrkB receptors – act on the assembly and maintenance of AChR clusters. CaMKII, a serine/threonine kinase activated by electrical activity, promotes insertion of AChRs into synaptic sites. Synapse-specific gene transcription, such as that mediated by the Ets transcription factor Erm, is important for AChR maintenance. By contrast, ephexin1-mediated upregulation of RhoA and activation of PKC and the actin modulator ADF/cofilin regulate AChR removal during plaque-to-pretzel transition. Cdk5-dependent dispersal of AChR clusters, enclosed in a dotted square, is a signaling pathway that has only been demonstrated in embryonic stages. In addition, dashed arrows indicate regulatory pathways established in vitro or in systems other than the NMJ. Abbreviations: Abl, v-abl Abelson murine leukemia viral oncogene homolog; ACh, acetylcholine; AChE, acetylcholinesterase; AChR, acetylcholine receptor; ADF, actin depolymerizing factor; APC, adenomatous polyposis coli; CaMKII, Ca²⁺/calmodulin-dependent kinase II; Cdk5, cyclin-dependent kinase 5; CK2, casein kinase 2; ColQ, collagen Q; DB, dystrobrevin; DG, dystroglycan; Dvl, dishevelled; Eph, erythropoietin-producing human hepatocellular carcinoma; ErbB, erythroblastic leukemia viral oncogene homolog; Erm, Ets-related molecule; GABP, growth-associated binding protein; GGT, geranylgeranyltransferase; JNK, c-Jun N-terminal kinase; LRP4, low-density lipoprotein receptor-related protein 4; MuSK, muscle-specific kinase; nNOS, neuronal nitric oxide synthase; NRG, neuregulin; NT-4, neurotrophin-4; NSF, N-ethylmaleimide sensitive factor; PDZRN, PDZ-domain-containing RING finger 3; PKC, protein kinase C; PTP, protein tyrosine phosphatase; SFKs, Src-family kinases; Tid1, tumorous imaginal disc 1; Trk, tropomyosin receptor kinase (Shi et al., 2012).

a. The AChR structure

The nicotinic AChR receptor ion channel is a pentamer composed of four subunits; 2 α 's, 1 β , 1 γ or 1 ϵ , and 1 δ , each encoded by separate genes. During the latter stages of maturation, (after birth), the AChR receptors replace their γ subunit with the ϵ subunit, with the replacement of the γ subunit by ϵ seen as a gradual transcriptional decrease and increase respectively (Sanes and Lichtman, 1999). The functional importance of this exchange has been proposed to be related to the kinetics of AChR and/or for the structural maturation of the synapse (Sanes and Lichtman, 1999) (Figure 7).

There are currently two models that explain how these subunits form the ion channel:

1) The first model (“heterodimer” model) postulates that the subunits first post-translationally fold into their tertiary structures. Once folded, two α subunits each associate with γ or δ to assemble α/γ or α/δ heterodimers. Finally, the heterodimers come together with the β subunit to structure the $\alpha/\beta/\gamma/\delta$ pentamer (Green, 1999).

2) In the second model (“sequential” model”), the β , and γ subunits are assumed to post-translationally fold into their tertiary structures and quickly assemble into a heterotrimer with an unfolded α subunit. The α subunit fold into its tertiary structure only after it has been incorporated into the heterotrimer. Once α has obtained its correct tertiary structure, the δ subunit joins the complex to make the $\alpha/\beta/\gamma/\delta$ tetramers. The proper orientation of the tetramer exposes the ACh binding site on the α subunit recruiting the second α subunit to make $\alpha/\beta/\gamma/\delta$ pentamers. The second ACh site forms on the pentamer shortly after (Green, 1999).

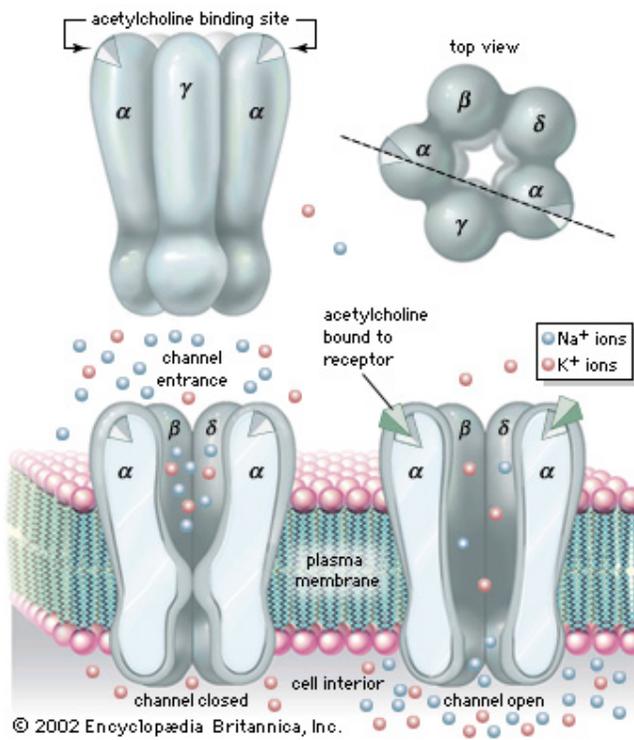


Figure 7. The structure of AChR. The AChR is a pentamer composed of two α subunits, a β subunit, a δ subunit and an embryonic γ subunit which is replaced shortly after birth by the adult ϵ subunit. The binding of two ACh molecules to their sites present on the N-terminal domain of the α subunits AChR, the channel formed by the hydrophobic transmembrane region of the five AChR subunits opens to let in sodium and calcium ions and potassium ions out.

b. Biosynthetic and secretory pathways of AChR

AChR subunit folding and pentamer formation occurs with the help of multiple chaperones in the endoplasmic reticulum (ER). Once inserted in the ER membrane, AChR pentamers are recruited to the ER exit site where they pass a quality control checkpoint. These checkpoints recognise specific retention and retrieval signals visible in misfolded subunits or improperly formed pentamers and target them for degradation. For example, each AChR subunit has a retention signal conserved in its transmembrane domain. This retention signal is not detectable if the subunits are properly assembled into a pentamer. The α subunit also contains a di-basic retrieval signal in its cytoplasmic loop which is exposed in unassembled pentamers. In accurately formed pentamers or when the subunit is bound to chaperones this sequence is no longer visible. Once properly oriented, AChR pentamers pass the ER checkpoint, then are transported by COPII vesicles to the Golgi and on to the muscle membrane (Colombo et al., 2013).

Once in the muscle membrane, AChR turnover is dependent on synaptic activity, as AChR membrane half-life is drastically decreased from 10-14 days to a few hours upon synaptic blockage. The stabilisation effect of synaptic activity on AChR has been proposed to be post-translationally regulated by the increases in calcium influx and cAMP (Pires-Oliveira et al., 2013).

AChR turnover at the membrane is primarily regulated by endocytosis via clathrin dependent and non-clathrin dependent mechanisms. However, the details of this mechanism are still unknown. Once internalized in the early endosome, AChR is either targeted to the lysosome where it is degraded via the ubiquitin-proteasome system or recycled back to the muscle membrane in an activity dependent manner (Pires-Oliveira et al., 2013). The dynamics of AChR at the NMJ discussed above is reviewed in figure 8.

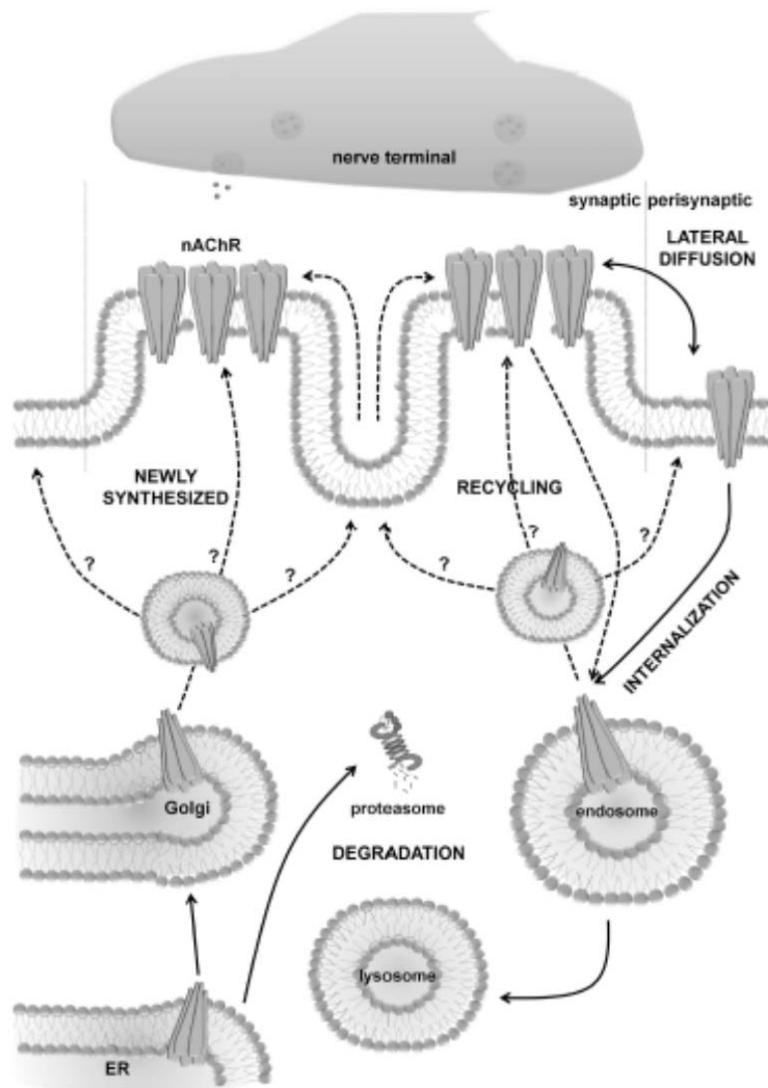


Figure 8. Dynamics of nicotinic acetylcholine receptors at the neuromuscular junction.

Nicotinic acetylcholine receptors (nAChR) subunits are synthesized in the ER and exported to the muscle plasma membrane. From the ER, instead of being targeted to the cell surface, most nAChR subunits are degraded by the ER-associated ubiquitin-proteasome degradation pathway. In the postsynaptic membrane, there is significant lateral diffusion between the synaptic and perisynaptic membrane spaces. Lateral diffusion of nAChRs from the perisynapse into the NMJ contributes significantly to maintain the synaptic receptor density. Conversely, when receptors escape from the postsynaptic density into the perisynaptic space,

there is significant internalization of nAChRs into endosomal compartments. Trafficking through the endosomal pathway, a fraction of internalized nAChRs is targeted for degradation. However, a significant portion of those nAChRs actually recycle back into the synaptic membrane, contributing to the maintenance of the synaptic nAChR pool. Most of these dynamics are tightly regulated in the NMJ by several stimuli, such as synaptic activity or association of dystrophin glycoprotein complex components (Pires-Oliveira et al., 2013).

c. MuSK regulates AChR levels at the NMJ

i. MuSK structure and activity

MuSK is indispensable for the formation of the NMJ as it serves as a multifunctional hub orchestrating the successive steps of synapse formation (Glass et al., 1996; Kim and Burden, 2008; Weatherbee et al., 2006). Its extracellular domain contains three immunoglobulin-like motifs and a cysteine-rich region called C6 (DeChiara et al., 1996). The C6 region is homologous to a sequence present in the Frizzled receptors and known to bind Wnt proteins. The extracellular domain of MuSK also forms a macrocomplex with LRP4, via its first immunoglobulin-like domain (Kim et al., 2008b; Zhang et al., 2008). The intracellular domain of MuSK contains a juxtamembrane domain that autophosphorylates in the presence of agrin, and a kinase domain. MuSK contains nineteen possible phosphorylation sites (tyrosine residues) and it has been shown that six of these sites must be phosphorylated in order for MuSK to be active (Watty et al., 2000)

MuSK intracellular co-receptor, Dok7 is both a substrate and activator of MuSK. Dok7 is an adaptor protein that contains an N-terminal pleckstrin-homology (PH) domain, a phosphotyrosine binding (PTB) domain and a C-terminal region containing sites of tyrosine phosphorylation (Beeson et al., 2006; Cai et al., 2003). The autophosphorylation of MuSK at Ty553 located in an NPXY sequence motif induces its binding to Dok7 through Dok7's PTB domain (Okada et al., 2006).

MuSK is activated by agrin binding to its extracellular domain, which helps to strengthen the LRP4/MuSK interaction. Agrin signalling is passed through the membrane to the intracellular domain where the MuSK/Dok7 interaction results in the phosphorylation of MuSK. The phosphorylation of MuSK results in its endocytosis via clathrin coated vesicles with Dok7 localizing MuSK in the caveolin-specific structures. The internalization of MuSK is required for AChR clustering (Luiskandl et al., 2013). Together the phosphorylation and endocytosis

of MuSK results in its activation and further induction of an amplitude of downstream signaling pathways, and interactions with scaffolding proteins and transcription of postsynaptic proteins (Wu et al., 2010).

ii. AChR clustering via MuSK and rapsyn

The agrin dependent activation of MuSK induces AChR clustering through a signaling cascade leading to the phosphorylation of the AChR β subunit at its Tyrosine Y390 and the recruitment of the cytoplasmic membrane associated protein, Rapsyn (Borges and Ferns, 2001; Borges et al., 2008). Rapsyn contains a myristoylation site necessary for its submembrane localization, seven tetratricopeptide repeats (TRPs), a putative coiled-coil domain, and a RING-H2 domain (Ramarao et al., 2001). Rapsyn is capable of interacting with itself through its TRP domain, with AChR by its coiled-coil domain, and to the actin cytoskeleton through its RING-H2 domain. Once at the post-synaptic membrane, rapsyn clusters phosphorylates AChR receptors through the interaction with the intracellular loop of AChR and the formation of small rapsyn bridges, with each receptor capable of binding three bridges (Ramarao et al., 2001; Zuber and Unwin, 2013).

The agrin/MuSK signaling pathway stabilizes the AChR clusters by inhibiting the ACh induced dispersion of the AChR clusters. It has been shown that ACh negatively regulates AChR cluster size through the activation of the cytoplasmic serine/threonine kinase cdk5 (Fu et al., 2005; Lin et al., 2005). Furthermore, in the absence of agrin, cholinergic stimulation activates calpain, a member of the calcium-activated intracellular cysteine protease family. Calpain cleaves p35 to p25 which goes on to activate cdk5. The presence of agrin inhibits the cholinergic dependent activation of calpain by inducing its interaction with rapsyn (figure 9) (Chen et al., 2007).

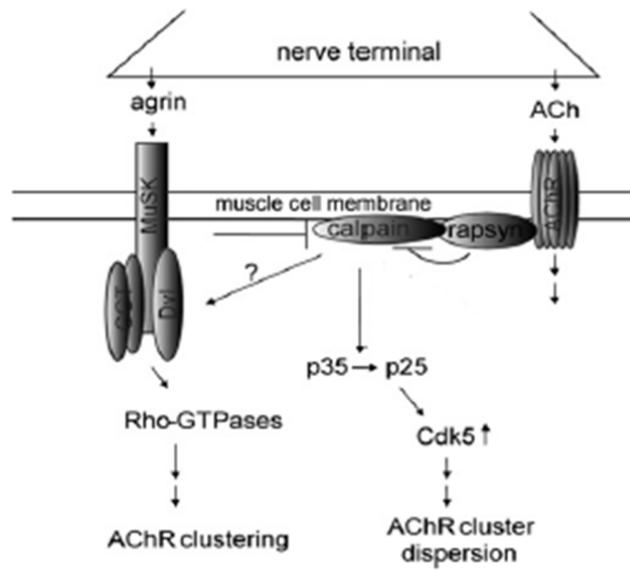


Figure 9. *The Working Hypothesis ACh activates Cdk5 in a manner dependent on calpain. Activated Cdk5 disperse AChR clusters. Agrin increases the interaction of rapsyn with calpain and thus inhibits calpain activity to stabilize AChR clusters at synapse (Chen et al., 2007).*

d. The control of AChR gene expression

Synapse formation induces an increase in the levels of AChR and other post-synaptic proteins. Post-synaptic gene expression is thought to be induced by the mitogen-activated protein kinase (MAPK), c-jun NH₂-terminal kinase (JNK) and the Phosphatidyl Inositol-3 kinase (PI3K) signaling pathways. These pathways converge to phosphorylate and activate the Ets family transcription factor, GA binding protein (GABP) *in vitro*. Phosphorylated GABP binds an N-box (CCGGAA) in the promoters of synaptic genes inducing their transcription (Schaeffer et al., 2001). Another member of the Ets family, Erm, has also been involved in the regulation of subsynaptic gene expression *in vivo* (Hippenmeyer et al., 2007) but only GABP was been shown to bind an N-box in the AChR subunit promoters, inducing their transcription in synaptic nuclei (figure 10) (Schaeffer et al., 2001; Schaeffer et al., 1998).

It has been well accepted that AChR gene expression is triggered by the previously described agrin/MuSK complex and by the neuregulin/Erb complex. At the NMJ two isoforms of Neuroregulins (NRGs) are present, *nrg-1* present in both the motor neuron and muscle fiber and *nrg-2* present in the terminal Schwann cells. NRGs activate the receptor tyrosine kinase ErbB family. These include ERbB2, ERbB3, and ERbB4. ERbB4 binds and activates NRG. ERbB2 cannot bind to NRG but possess tyrosine kinase activity while ERbB3 binds NRG but lacks catalytic activity. Therefore, ERbB2 and ERbB3 can only be NRG activated as heterodimers with other ERbBs (Rimer, 2007). Muscle fibers express all three ERbB receptors while terminal Schwann cells express only ERbB2 and ERbB3 (Rimer, 2007).

NRG has been widely accepted to bind ErbB receptors and stimulate synaptic gene transcription. However, these conclusions were all based on *in vitro* studies because knock-out mice died before birth (Chu et al., 1995; Falls et al., 1993; Gramolini et al., 1999). Since 2005, two groups using conditional knock-out mice have shown that NMJs can form in the

absence of muscle specific ErbB2 and ErbB4 and even went further to demonstrate that post-synaptic differentiation is induced by agrin (Escher et al., 2005). In 2006, Jaworski and Burden inactivated *nrg-1* in the motor neuron and skeletal muscle first separately then simultaneously, and found that the AChR clusters still formed at the synapse and that synapse-specific transcription was normal (Jaworski and Burden, 2006). Thus the role of NRGs in synaptic gene transcription has yet to be clarified *in vivo* versus *in vitro*.

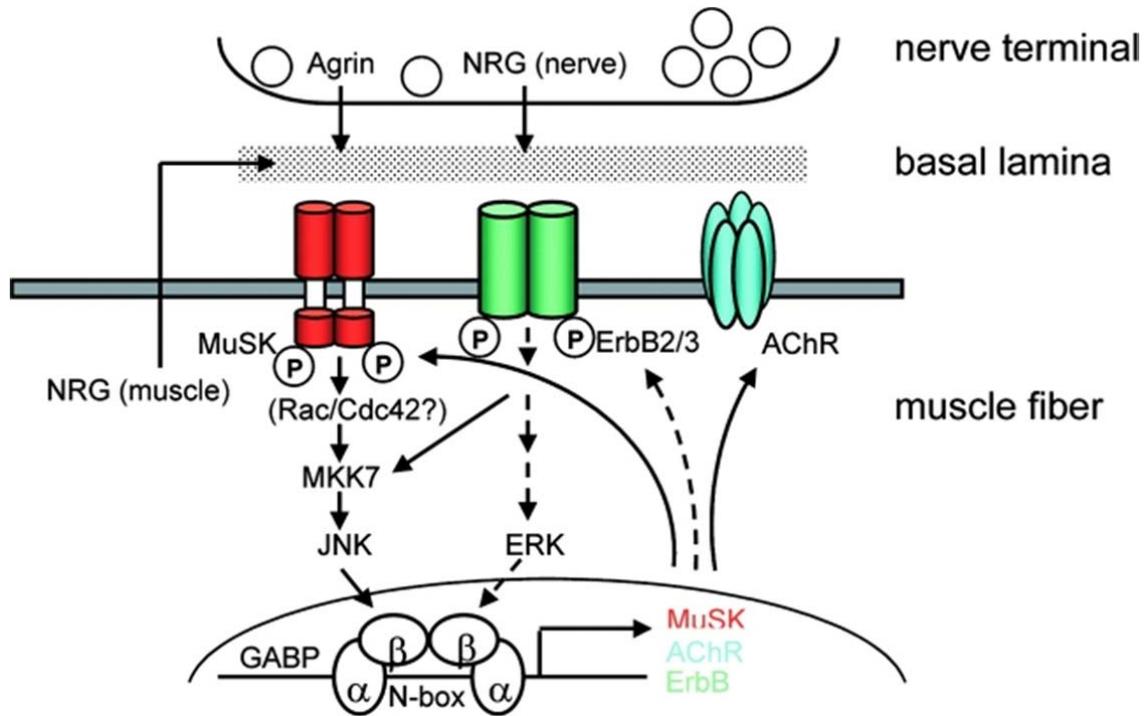


Figure 10. Model for stabilization of synaptic gene expression through stabilization of musk expression by agrin from motor nerve terminal. Agrin secreted from nerve terminal activates preexisting MuSK to induce expression of musk via its N-box (i) by organizing an NRG/ErbB pathway, involving MuSK-induced recruitment of ErbB receptors and of muscle-derived NRG and (ii) by MuSK-induced activation of JNK (via Rac/Cdc42). With musk expression stabilized, the same pathways are used for AChR and erbB expression. Expression may be strengthened by NRG-1 secreted from nerve terminal. Complete inhibition of Agrin-induced musk transcription in C2C12 cells by overexpression of inactive ErbBs (HER2KM and HER4KM) and by dominant-negative JNK suggests that the two pathways are connected. The model is based on present data (solid arrows) and references cited (broken arrows) (Lacazette et al., 2003).

e. Pathologies linked to AChR deficiency

Congenital myasthenic syndromes (CMS) correspond to a class of human pathologies resulting from mutations in genes coding for proteins involved in NMJ activity, structure, or development. Mutations in AChR subunits were the first to be studied in patients with CMS. These studies focused on subunit mutations that impaired channel gating or that decreased AChR levels at the membrane. Scientists were able to separate 2 classes of heterozygous recessive mutations: those that alter the kinetic properties of AChR and those that reduce the expression of AChR. The latter group of mutations can be further classified into “slow channel” mutations and “fast channel” mutations (Engel and Sine, 2005).

Slow channel mutations are dominant gain of function mutations that increase ACh response resulting in the slow decay of synaptic current. This gain of function mutation can occur via two mechanisms. The first being the abnormally slow closure of activated receptor channels and the second being ACh bound receptor channels having an increased probability of opening. Thus both mechanisms increase the probability of open AChR channels (Engel and Sine, 2005).

Contrary to slow channel mutations, the fast channel mutations decrease synaptic response to ACh by decreasing the probability that AChR channels will be open, resulting in an abnormally fast decay of synaptic response. The reduced activation of AChR results from mutations which cause the channel to close abnormally fast and those that result in ACh bound channels closing abnormally slow. Fast channel mutations cause the synapse to have brief current pulses followed by prolonged intervals between pulses (Engel and Sine, 2005).

The incomplete atomic structure of AChR has made it difficult to understand how mutations in the AChR subunits affect its kinetics. However, the recent crystallization of ACh structure provided insight into the characteristics of its ligand binding site on AChR (Brejc et al., 2001). This was then fused together with the atomic structure of the pore from the torpedo

and a recent density electron map providing almost the whole AChR receptor (Bouzat et al., 2004; Miyazawa et al., 2003; Unwin, 2005). This has allowed for the mapping of the CMS mutations from which they have determined that mutations in the ACh ligand binding site alter the channel kinetics, while mutations in the pore domain alter the gating of AChR.

CMS with AChR deficiency is not only encountered upon mutations in AChR subunits but also has been found to be induced by mutations in Rapsyn, MuSK and Dok7 all of which reduce AChR clustering at the post-synaptic membrane (Engel and Sine, 2005).

2. THE MATURATION AND MAINTENANCE OF THE NMJ

A. Presynaptic Maturation

1. Synapse elimination

In neonatal animals, each muscle fiber is innervated by multiple motor axons, whereas in the adult only one motor axon is present at the mature NMJ (Figure 11). This developmental phenomenon is known as synapse elimination and its occurrence is comprised in rodents between the time of birth and postnatal day 15 (P15), with a few days' variation in specific muscles. The number of motor neurons in the spinal cord remains constant during this developmental period. Synapse elimination is therefore a peripheral process that only involves motor nerve endings and their axonal branch, occurring over several days, with a percentage of fibers becoming singly innervated each post-natal day.

Synapse elimination occurs in 4 steps: axonal atrophy, axonal detachment and the formation of the retraction bulb, and axonal withdrawal from the endplate. Through synapse elimination and post-natal presynaptic maturation, each motor unit (the muscle fibers and their innervating motor neuron) undergoes a change from a large size, overlapping with other units, to a much smaller, non-overlapping one, which is retained as a permanent feature throughout adult life (Buffelli, 2004).

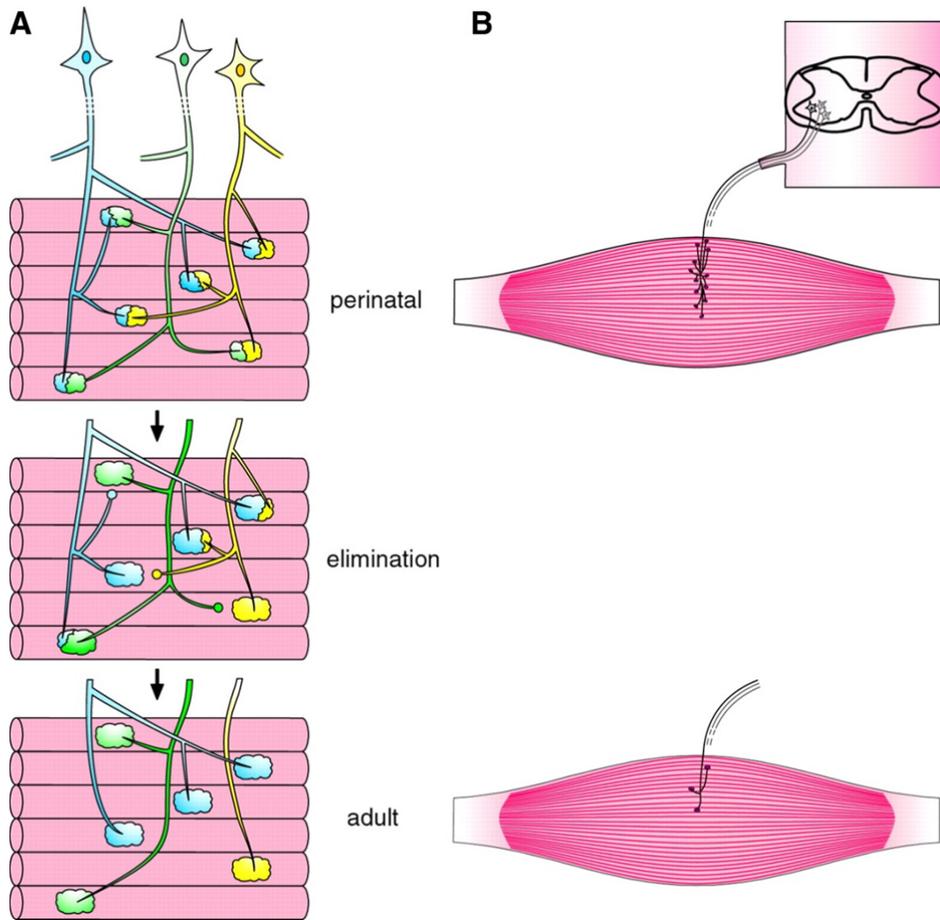


Figure 11. Synapse elimination. Neonatal synapse elimination represented as the loss of polyneuronal innervation (A) and the reduction in motor unit size (B) (Buffelli, 2004).

2. The interplay between the motor neuron and the Schwann cells

Motor neuron maturation depends highly on that of the maturation of Schwann cell and vice versa. Schwann cells have been known to generate diffusible signals such as TGF β , which promote the development and function of the motor neuron. Schwann cells also engulf and clean up axons that become fragmented during synapse elimination.

Equally, Schwann cell survival is dependent on signaling from the motor neuron, specifically, the release of neutrophin 3, which regulates the number of Schwann cells and myelinating glial cells present at the NMJ. Further, the motor neuron also releases NRG 1 which promotes Schwann cell survival and development (Wu et al., 2010).

3. Pathways regulating presynaptic maturation

Most of the key players in the NMJ formation are found on the post-synaptic muscle and the pathways used to organize the post-synaptic differentiation have been largely studied. It seems as though these post-synaptic molecules also act in retrograde to control presynaptic differentiation and maturation, as independently knocking out MuSK, LRP4, or Agrin results in the loss of presynaptic differentiation (DeChiara et al., 1996; Kim et al., 2008b; Zhang et al., 2008).

What we do know is that the accuracy of protein turnover appears to be important in presynaptic differentiation. Ubiquitin specific peptidase 14 (Usp14) is a proteasomal deubiquitinating enzyme, which when knocked out, results in the accumulation of phosphorylated neurofilaments and nerve terminal sprouting (Chen et al., 2009). Furthermore, ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1), also a deubiquitinating enzyme, is needed for accurate synaptic vesicle accumulation and the turnover of tubulovesicular structures at the presynaptic terminal (Chen et al., 2010). Therefore, it

appears that the ubiquitin processing system is involved in the development of the presynaptic terminal through the targeting of proteins for degradation and clearance.

B. Post-Synaptic Maturation

Structural and functional maturation of the post-synaptic membrane during development controls the association and refinement of neural circuits. Post-synaptic maturation includes three main processes: 1) The change in morphology, composition, and stability of AChR cluster, 2) The formation of membrane invaginations called junctional folds, and 3) the reorganization of the cytoskeleton. In the following paragraphs, each of these processes will be described in further detail.

1. Transformation of AChR clusters during NMJ maturation.

The initial formation of the NMJ is marked by the presence of a dense oval-like structure of AChR clusters resembling a uniform plaque. During the first three post-natal weeks, selective regions of this plaque disassemble to generate multi-perforated branches that resemble a pretzel-like structure (Figure 12A). As the muscle fibers grow length wise, the AChR clusters continue to grow, with the initial plaque-like structure integrating new receptors in the peripheries of the clusters. As the pretzel shape takes form, AChR cluster size and branch shape remains the same, however their density increases as new receptors are added within the clusters. Furthermore, as the NMJ matures the half-life of the receptors increase and are more resistant to disassembly (Shi et al., 2012). The life time of AChRs in the membrane increases from less than one day for embryonic clusters to greater than one week in postnatal life (Fambrough, 1979).

The adult AChR clusters differ from its neonatal counterparts molecularly as well as topologically, with the most well-known molecular change being that of the subunit

composition. The embryonic form of AChR which contains the γ subunit is replaced by that of the homologous ϵ subunit within the first week after birth. This γ -to- ϵ switch is known to occur at the transcriptional level and has two effects on AChR conductance: the shortening of the duration the receptor channel opening and the increasing calcium permeability of the channel (Sanes and Lichtman, 2001). The γ -to- ϵ switch is also important for synaptic maintenance of AChR clusters and the development of junctional folds (Missias et al., 1997; Schwarz et al., 2000).

2. The formation of junctional folds

The efficient neurotransmission of the NMJ is dependent on the structural topology of the post-synaptic membrane. As the membrane begins to mature it sinks to form gutters called the primary fold, which then elongate to form parallel secondary folds called junctional folds. These folds are spaced at regular intervals and are oriented orthogonally to the long axis of the muscle fiber (Figure 12B). The formation of the junctional folds has multiple functional importance. First, it allows for the enrichment of AChR ($10,000/\mu\text{m}^2$) at the crest of the junctional folds by placing them in close proximity to ACh release sites. Secondly, it segregates AChR from the sodium voltage dependent channels concentrated in the depths of the junctional folds. This provides a mutually exclusive arrangement with AChR and the sodium channels, which is considered to amplify the synaptic current by causing a voltage drop across the electrical resistance of the interfolds. Lastly, the junctional folds form subdomains which serve as platforms for postsynaptic receptors and membrane associated proteins (Shi et al., 2012). The same holds true for constituents of the basal lamina. Laminin $\alpha 2$, $\alpha 5$, and $\beta 2$ are present in the synaptic clefts and folds while laminin $\alpha 4$ is present only in the synaptic clefts (Patton et al., 2001).

3. The organization of the synaptic cleft

The synaptic cleft spans about 50-100 nm and contains a basal lamina made up of a multitude of large molecules, forming an extracellular matrix that aids cell adhesion and appropriate neuromuscular signaling processes. The synaptic basal lamina is composed of a distinct set of ECM proteins which extend through the synaptic cleft into the junctional folds. They are of 4 main types: Heparan sulfate proteoglycans, such as the previously described neural agrin and laminins, collagens, and nidogens which are synthesized and secreted by the muscle. It should be noted that most of the ECM components are also present in the extrasynaptic areas but some isoforms are largely enriched at the synapse. These components not only provide the lamina with structural support by forming intra-networks and links to neighboring structures, but they are also signal molecules that activate signal-transducing receptors in the pre- and postsynaptic membranes and strengthen ligand binding to the receptors.

The NMJ basal lamina also contains nerve and muscle derived signaling molecules, such as acetylcholinesterase (AChE), agrin, and neuregulins, which all participate to ensure effective neurotransmission (Sanes and Lichtman, 2001). The following sections will describe the functional role of the 3 well characterized ECM proteins, Laminins, Collagens, and Perlecan.

a. Laminins

Three laminin heterodimers are present at the synaptic BL and regulate maturation: laminin 221($\alpha 2\beta 2\gamma 1$), 421($\alpha 4\beta 2\gamma 1$), and 521 ($\alpha 5\beta 2\gamma 1$) (Patton et al., 1997). Laminin $\beta 2$ has previously been described for its importance in active zone formation through its binding to presynaptic Calcium voltage gated channels. Moreover, Laminin $\beta 2$ also aids in nerve terminal differentiation by inhibiting the excessive outgrowth of terminal branches. Postsynaptically, Laminin $\beta 2$ guides the development of junctional folds and the topological maturation of AChR clusters (Shi et al., 2012). Furthermore, knock-out studies in mice have shown that Laminin 421 and 521 aids in the plaque-to-pretzel transition of AChR (Nishimune

et al., 2008; Patton et al., 1997). Likewise, Laminin 421 also aids in the alignment of the presynaptic active zone and the opening of the post synaptic junctional folds (Patton et al., 1997).

b. Collagens

Three main types of Collagens are present in the synaptic basal lamina whose functions have been derived from the phenotypic assessment from knock-out studies:

- i. Collagen IV chains ($\alpha3$ - $\alpha6$) are expressed in the mature NMJ and are essential for the maintenance of the adult motor neuron terminals (Fox et al., 2007).
- ii. Collagen XIII is a transmembrane collagen expressed by the muscle. Collagen XIII is concentrated at the postsynaptic membrane but its ectodomain can be proteolytically cleaved and released into the extracellular matrix. Presynaptically, this collagen regulates the opposition of the active zone with the junctional folds. Post-synaptically, Collagen XIII is essential for the topological maturation of AChR clusters (Latvanlehto et al., 2010).
- iii. Collagen Q (ColQ) is a synaptic collagen which anchors AChE to the post-synaptic membrane (Krejci et al., 1999; Krejci et al., 1997) and thus indirectly regulates ACh concentrations in the synaptic ECM. ColQ is accurately positioned at the postsynaptic membrane through its interaction with MuSK and Perlecan, a heparan sulfate proteoglycan (Cartaud et al., 2004; Kimbell et al., 2004).

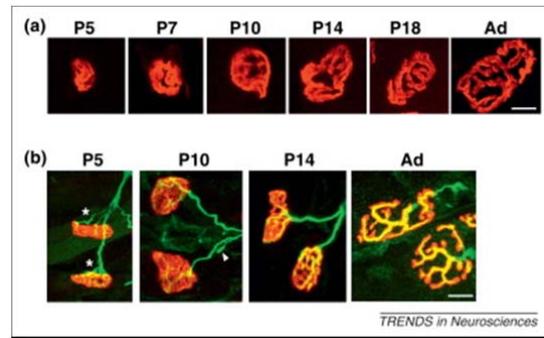
c. Perlecan

Perlecan is the heparin sulfate proteoglycan found in most basement membranes, with the physiological importance of perlecan being highlighted by the neonatal lethality of perlecan null mice (Arikawa-Hirasawa et al., 1999). Perlecan is widely expressed in basement membranes, providing mechanical strength through the stabilization of the laminin and Collagen IV network and aids in cell signalling via its binding to α Dystroglycan, integrins, and growth factors (Costell et al., 1999). The absence of functional perlecan or hypomorphic

mutations in the human perlecan gene are the cause of two rare autosomal recessive diseases, Dyssegmental Dysplasia, (Silverman-Handmaker type; DDSH) and Schwartz Jampel Syndrome (SJS) (Stum et al., 2005). Model mice for these two pathologies have been generated and stress the dosage effect of perlecan (Arikawa-Hirasawa et al., 1999; Stum et al., 2008).

Specifically at the NMJ, perlecan anchors the ColQ/AChE complex to the synaptic basal lamina (Arikawa-Hirasawa et al., 2002). However, the importance of perlecan at the NMJ appears to be beyond that of its role in ColQ/AChE as it has also been shown to play a role in Schwann cell physiology (Bangratz et al., 2012).

A.



B.

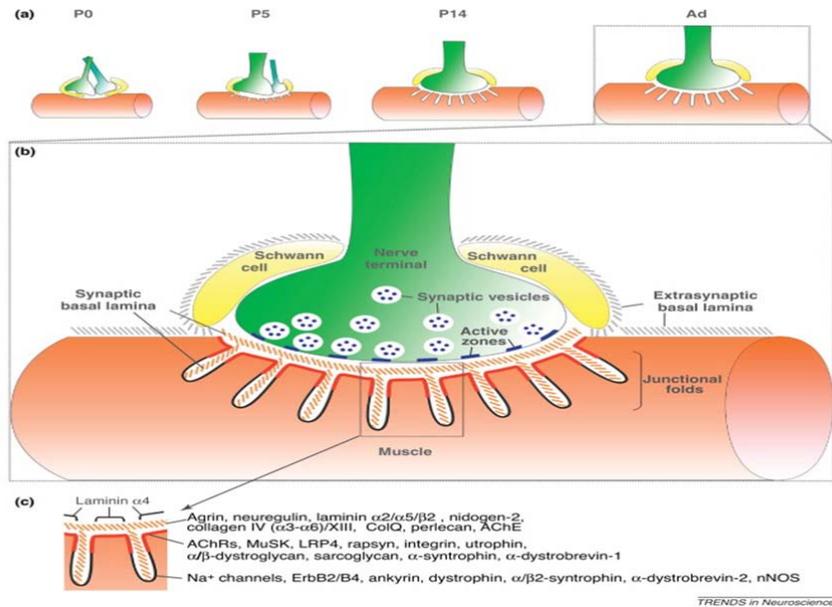


Figure 12. The postnatal maturation of the mouse NMJ.

A. Morphological changes in the mouse NMJ during postnatal development. (a) The morphology of early postnatal AChR clusters appears as an oval-like plaque. Within the first three postnatal weeks, AChR plaque is transformed into a multiperforated array of branches that has a pretzel-like shape. P, postnatal day; Ad, adult. (b) During the first two postnatal weeks, elimination of axonal inputs occurs at the initial poly-innervated endplates (asterisks), transforming them into singly innervated (the retraction bulb of an eliminated axon is indicated by an arrowhead). Furthermore, the axon terminals differentiate to perfectly align with the AChR cluster branches. Photomicrographs show examples of NMJs at the tibialis anterior muscle from ICR mice during the postnatal stages indicated. Postsynaptic AChR clusters and presynaptic nerve terminals are labeled by α -bungarotoxin (BTX; red) and synaptophysin/neurofilament (green), respectively. Scale bars, 10 μ m (Shi et al., 2012).

B. Schematic illustrating development of the vertebrate NMJ, with a particular emphasis on junctional folds. (a) Formation of synaptic gutters and junctional folds during postnatal maturation of NMJ. P, postnatal day; Ad, adult. (b) An adult vertebrate NMJ. (c) Molecules expressed at the tops and troughs of adult junctional folds, as well as those expressed at the synaptic basal lamina. Such molecules include glycoproteins (e.g., laminins), receptors (e.g., MuSK and ErbB receptors), and scaffolding proteins (e.g., dystrophin, syntrophins, and utrophin) (Shi et al., 2012).

C. NMJ Function: From an Action Potential to a Muscle Contraction

The nerve terminal is responsible for neurotransmitter release into the synaptic cleft. It is thus the site for: 1) neurotransmitter synthesis and its incorporation into vesicles, 2) neurotransmitter release and its reuptake and 3) the transportation of ions across the nerve terminal cell membrane. In the vertebrate NMJ, the neurotransmitter released is ACh, which is synthesised in the nerve terminal from acetyl coenzyme-A and choline by the enzyme choline acetyltransferase. Thousands of ACh molecules are then packed into each vesicle through an energy dependent vesicular transport process and stored in two major sites: at the active zone near the presynaptic membrane or in a remote position where they await their transport via the cytoskeleton down to the active zone for release (Fagerlund and Eriksson, 2009).

When the motor neuron receives a signal, it sends an action potential to the distal nerve terminal. This induces the voltage gated Calcium channels, which are in high concentration at the terminal, to open thereby generating an inward Calcium flux. The resulting increase in intracellular Calcium concentrations activates the membrane associated protein, synaptotagmins, which binds and mediates the formation of the SNARE (soluble NSF attachment receptor proteins) protein complex. Together with the SNARE complex, SM proteins (Sec1/Munc18-like proteins) form the core fusion machinery for the docking of the ACh containing vesicles at the active zone. The active zone protein complex containing RIM (Rab3-interacting molecule), Munc13, RIM-BP (RIM-interacting molecule), and a Calcium channel in the presynaptic plasma membrane interact with the SNARE complex in further vesicular release. This protein complex binds to specific target proteins to mediate vesicle docking, priming, and the recruitment of the calcium channels within 100 nm of the docked vesicles for fast excitation-secretion coupling (Sudhof, 2013). Once ACh molecules are released, the used vesicles are recycled by active transport into the nerve terminal, where they

are reused to form new neurotransmitter packed vesicles (Fagerlund and Eriksson, 2009)
(Figure 13).

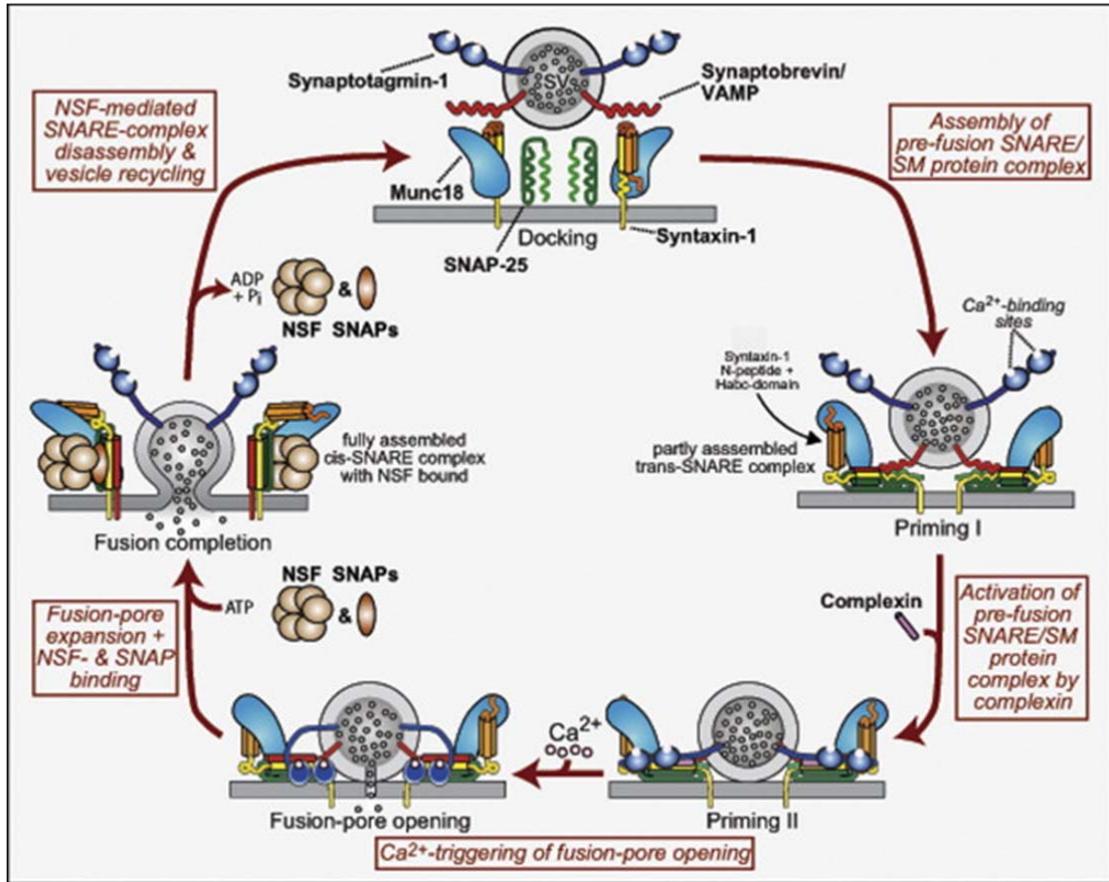


Figure 13. Schematic of the SNARE/SM Protein Cycle Mediating Fusion and the Role of Synaptotagmin and Complexin in Ca²⁺ Triggering of Fusion.

SNARE and SM proteins undergo a cycle of assembly and disassembly, such that the vesicular SNARE protein synaptobrevin assembles during priming into a trans-SNARE complex with the plasma membrane SNARE proteins syntaxin-1 and SNAP-25. Prior to SNARE complex assembly, syntaxin-1 is present in a closed conformation in which its Habc domain folds back onto its SNARE motif; this conformation precludes SNARE complex assembly, and syntaxin-1 has to “open” for SNARE complex assembly to initiate. Moreover, prior to SNARE complex assembly, Munc18-1 is associated with monomeric syntaxin-1 when syntaxin-1 is in a closed conformation; as syntaxin-1 opens during SNARE complex assembly, Munc18-1 alters the mode of its binding to syntaxin-1 by binding to assembling trans-SNARE complexes via interacting with the syntaxin-1 N-peptide. Once

SNARE complexes are partially assembled, complexin binds to further increase their priming. The “superprimed” SNARE/SM protein complexes are then substrate for Ca²⁺-triggered fusion pore opening by Ca²⁺ binding to synaptotagmin, which causes an interaction of synaptotagmin with SNAREs and phospholipids. However, even before Ca²⁺ triggering, synaptotagmin likely at least partly interacts with the fusion machinery as evidenced by the unclamping of spontaneous mini release in Syt1 knockout neurons. After fusion pore opening, the resulting cis-SNARE complexes are disassembled by the NSF/SNAP ATPases, and vesicles are recycled, refilled with neurotransmitters, and reused for release (Sudhof, 2013).

Once in the synaptic cleft, ACh binds to the α subunit of the AChR on the post synaptic muscle membrane. However, not all ACh molecules reach the receptors because they are either quickly hydrolysed back to acetyl coenzyme-A and choline by AChE, which are anchored in the synaptic cleft by ColQ, or diffuse out of the synaptic cleft. Two molecules of ACh bind AChR at its high affinity binding site located between AChR subunits α and δ and its low affinity site located between subunits α and γ/ϵ . The binding of ACh to AChR results in its conformational change, opening its cationic canal. The large size of the canal permits the entry of sodium ions and the output of potassium ions with a net influx of sodium ions.

The summation of AChR openings leads to a localized depolarization that spreads passively along the muscle. Once this depolarization reaches a threshold, called the end plate potential, it induces the activation of voltage dependent sodium channels in the depth of the folds. This zone contains a high density of Sodium channels capable of amplifying the response to membrane depolarization. As a result, the perijunctional zone promotes the transduction process leading to muscle contraction by converting the end-plate potential into an action potential. Finally, muscle contraction is terminated gradually as ACh molecules diffuse or are degraded and the AChR receptors close their canals and are no longer able to form endplate potentials (Kandel et al., 2013).

IV. COLQ AND MYASTHENIC SYNDROME

A. ColQ Structure and Function

1. The origin of ColQ

CollagenQ (ColQ) is a rather ubiquitous collagen expressed in cholinergic tissues such as the brain, muscle and heart and non-cholinergic tissues such as the lung and testis (Krejci et al., 1997). Except for the lungs, ColQ's tissue distribution is identical to that of acetylcholinesterase (AChE) (Krejci et al., 1999; Krejci et al., 1997).

The first evidence of the existence of ColQ came from the identification of a specific form of AChE containing a collagen isolated from the torpedo californica electric organ and then in the electric eel, (Anglister and Silman, 1978; Lwebuga-Mukasa et al., 1976). In both animal species, the main form of AChE contains hydroxylysine and hydroxyproline, two amino acids over-represented in collagens. Electron microscopy provided better insight into this AChE form, demonstrating that tetramers of AChE subunits are attached to a rodlike tail and form an asymmetric structure (Cartaud et al., 1978). When the molecular and structural analysis was brought together, it suggested the existence of catalytic subunits linked to a collagen tail. This molecular form of AChE was termed the asymmetric form (A form) or the AChE tailed-form. Furthermore, the A form was found to be associated to synaptic basal lamina in torpedo (Lwebuga-Mukasa et al., 1976) and mammals (McMahan et al., 1978), suggesting that the collagen tail acts as an anchor to AChE.

The primary sequence of this collagen was elucidated in the 1990's first in torpedo (Krejci et al., 1991) then in rat (Krejci et al., 1997), and was later termed ColQ in agreement with the international nomenclature for collagens.

2. The structure of ColQ

ColQ contains a central collagenous region flanked by N- and C-terminus non-collagenous peptides that are conserved in the mature protein. Besides the repetition of the Gly-X-Y characteristic motifs, the collagenic domain contains two heparin-binding sites (Deprez and Inestrosa, 1995). Following the collagen domain, the C-terminal region contains a subdomain that is responsible for the trimerization of the collagen tail (Bon et al., 2003). A polyproline stretch located in the N-terminus of ColQ, allows the covalent interaction with the C-terminus of AChE catalytic subunit. Three strands of ColQ associate in a triple helix and each trimer can bind one to three tetramers of cholinesterase (Dvir et al., 2004; Simon et al., 1998) (Figure 14A).

In skeletal muscle, ColQ anchors AChE in the synaptic basal lamina of the NMJ, where hetero-oligomers of AChE-ColQ are highly enriched, although the relative ratio between the synaptic and extrasynaptic expression varies according to muscle types (Krejci et al., 1999). At the NMJ, ColQ positions AChE in the synaptic cleft to control the amount of acetylcholine in this space.

The three main domains of ColQ interact with proteins present at the NMJ. Besides the interaction with AChE through its N-terminus, ColQ interacts with two other partners: perlecan, a heparan sulfate proteoglycan (Arikawa-Hirasawa et al., 2002), and MuSK (Cartaud et al., 2004), a muscle-specific transmembrane tyrosine kinase receptor. The central collagen domain contains two heparin-binding sites which are supposed to mediate the interaction with perlecan (Kimbell et al., 2004). Perlecan binds dystroglycan, a transmembrane protein that not only forms a scaffold with perlecan, but also plays an essential role in linking the extracellular matrix to the cytoskeleton (Jacobson et al., 2001; Peng et al., 1999). The C-terminus of ColQ binds MuSK (Cartaud et al., 2004; Kawakami et al., 2011). Both the C-terminal and heparin-binding domains of ColQ are essential for

anchoring AChE in the synaptic basal lamina (Kimbell et al., 2004). While perlecan is expressed along the muscle fiber, MuSK is only expressed at the NMJ and therefore the association between ColQ and MuSK dictates AChE synaptic localization.

3. Consequences of ColQ Deficiency for Muscle Activity

a. ColQ and congenital myasthenic syndromes

As previously stated, congenital myasthenic syndromes (CMS) correspond to a class of human pathologies resulting from mutations in genes coding for proteins involved in NMJ activity, structure, or development. These disorders are characterized by a dysfunction of the NMJ, leading to muscle weakness and excess fatigability, together with additional specific symptoms related to the mutated gene and its function (Hantai et al., 2013).

In 1977, a patient with a CMS associated with AChE deficiency at the NMJ was described for the first time (Engel et al., 1977) and since then, a number of patients with this similar type of CMS have been identified. CMS associated with AChE deficiency is known to be a rare autosomal recessive disease. AChE deficiency in this syndrome is not due to mutations in the *ACHE* gene (Camp et al., 1995) but to mutations in *COLQ* (Donger et al., 1998; Ohno et al., 1998). With over 30 identified to date (Figure 14B), mutations in the *COLQ* gene are spread throughout the different functional domains and include null alleles, missense or frameshift mutations, and deletions. In general, null alleles give a more severe phenotype than those of missense alleles (Wargon et al., 2012).

Mutations in the *COLQ* gene have been divided into 3 major types (Figure 14B): 1) *Abrogation of attachment of catalytic subunits*: These mutations are located in the PRAD domain and prevent the attachment of AChE to ColQ, 2) *Truncation mutants in collagen domain preventing triple helix formation*: Mutations in the Collagen Domain prevent the formation of the triple helix resulting in single stranded ColQ with one AChE tetramer. These

species cannot be inserted into the membrane and 3) *C-terminal mutation preventing triple helix formation*: Mutations occurring in the trimerization domain of the C-terminus hinder the triple helix formation of the collagens producing asymmetric ColQ/AChE complexes. These complexes are not inserted into the membrane. Mutations in C-terminus have recently been shown to affect ColQ's binding to MuSK (Arredondo et al., 2013; Ohno et al., 2000).

The first symptoms of the CMS with AChE deficiency are detected in the neonatal period or in childhood and most of the patients are severely disabled. For the majority of the patients, the clinical picture includes proximal and axial muscle weakness, ptosis, respiratory insufficiency and delayed motor development (Mihaylova et al., 2008; Wargon et al., 2012). Muscle weakness appeared to affect the proximal muscle more frequently than the dorsal muscle. Weakness in proximal muscle includes waddling gait, muscle atrophies, scapular winging and diminished reflexes (Mihaylova et al., 2008). The diagnosis of CMS with AChE deficiency is based on the presence of repetitive compound muscle action potential (CMAP) after single stimulation, slow response of the pupil to light, and absence of AChE visualization at the NMJ.

The therapeutic options for CMS with AChE deficiency is still poor, with a number of CMS patients receiving treatment with AChE inhibitors such as pyridostigmine. However, this treatment tends to worsen the symptoms in CMS patients with AChE deficiency (Lorenzoni et al., 2012). This is probably due to the fact that pyridostigmine is inhibiting the already low levels of AChE brought on by the ColQ mutation. On the other hand, two β 2-adrenergic receptor agonists, ephedrine and salbutamol that are mostly used as antiasthmatic drugs, have been found to be beneficial for patients with ColQ mutations (Chan et al., 2012; Eymard et al., 2013; Mihaylova et al., 2008). Currently there is no explanation for the mechanism of action for these compounds.

b. A mouse model of congenital myasthenic syndrome with AChE deficiency.

A mouse model of CMS with AChE deficiency has been generated by the knock-out of the COLQ gene (Feng et al., 1999). ColQ knock-outs were produced through the use of 2 target vectors which deleted the exon containing the PRAD domain along with adjacent intronic sequences. As expected, the NMJ of these mice are found to be devoid of AChE and ColQ mutant mice exhibit myasthenic syndromes. The mice present the characteristic features of the patients with muscle weakness, atrophy of the muscles, heterogenous diameters of muscle fibers, an increased ratio of slow to fast fibers, signs of muscle necrosis and mild muscle atrophy, an abnormal synaptic structure, and Schwann cell processes extending into the synaptic cleft (Feng et al., 1999; Sigoillot et al., 2010a) (Sigoillot et al., in preparation; see annex). Intravenous viral injection of ColQ cDNA in these mutant mice has been shown to restore a normal phenotype in the motor functions, synaptic transmission and structure of the NMJ (Ito et al., 2012). Thus, the ColQ mutant mouse is a valid model to decipher the roles of ColQ in synapse formation and maintenance, as well as to understand the pathology of CMS and the basic mechanisms of drugs that improve the myasthenic symptoms.

4. The Roles of ColQ

a. ColQ and postsynaptic differentiation

At first, ColQ serves as a structural element of the NMJ, anchoring AChE in the synaptic basal lamina. However, in addition to anchoring AChE, it was demonstrated that ColQ participates in the postsynaptic differentiation/plasticity by regulating the density of AChR and the size of the synaptic clusters (Sigoillot et al., 2010a; Sigoillot et al., 2010b).

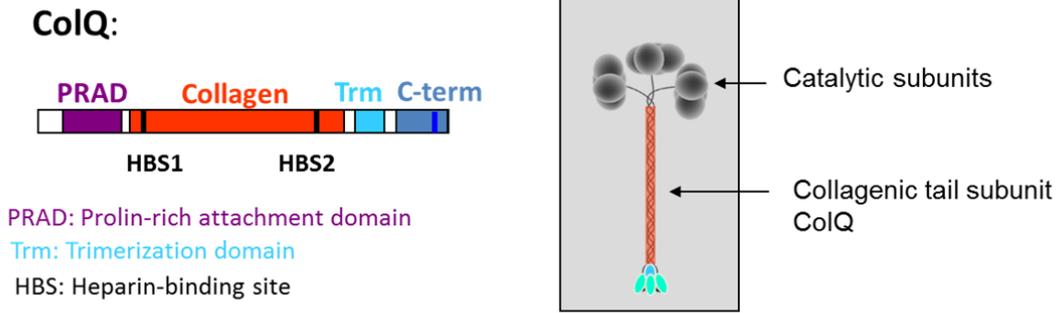
In ColQ knock-out mice, the surface of AChR clusters is smaller but their density is higher at the NMJ. This phenotype can be explained by molecular and subcellular events induced by the lack of ColQ. The absence of ColQ *in vivo* and *in vitro* induces an upregulation of

postsynaptic mRNAs including those coding for the AChR subunits, AChE, Rapsyn, and MuSK (Sigoillot et. al., in preparation; see annex). ColQ overexpression assays in MuSK null cells (provided by Ruth Herbst and Steve Burden) suggest that this effect is mediated by MuSK since AChR mRNA levels were not modified in these conditions. ColQ has also been found to regulate the levels of membrane-bound MuSK. We have demonstrated that ColQ deficiency leads to a reduced level of membrane-bound MuSK *in vitro* and *in vivo* (Sigoillot et al., 2010a), a process accompanied by a decrease in MuSK signaling.

b. ColQ regulates AChR expression

Most of the focus of synaptic AChR expression has been placed on its transcriptional regulation via two pathways triggered by Agrin and Neuregulin, two neurotrophic factors. However, several previous studies have implied the importance of mRNA stability in dictating the abundance of mRNAs in muscle (von Roretz et al., 2011). Amongst these studies, it has been shown that the ubiquitously expressed HuR, an mRNA binding protein plays a critical role in differentiating muscle cells. In support of a post-transcriptional regulation of AChR, preliminary experiments did not indicate a change in transcriptional activity of AChR subunit genes in the absence of ColQ. Furthermore, microarray studies completed in our laboratory showed that factors known to regulate the transcription of AChR are not differentially expressed in WT versus ColQ deficient muscle cells. Thus, these two studies suggest that ColQ may not be regulating AChR subunit mRNA at the transcriptional level. However, it should be noted that absence of ColQ may alter the activity of transcription factors through a post-translational modification, such as phosphorylation, and thus this would not be apparent in the microarray.

A.



B.

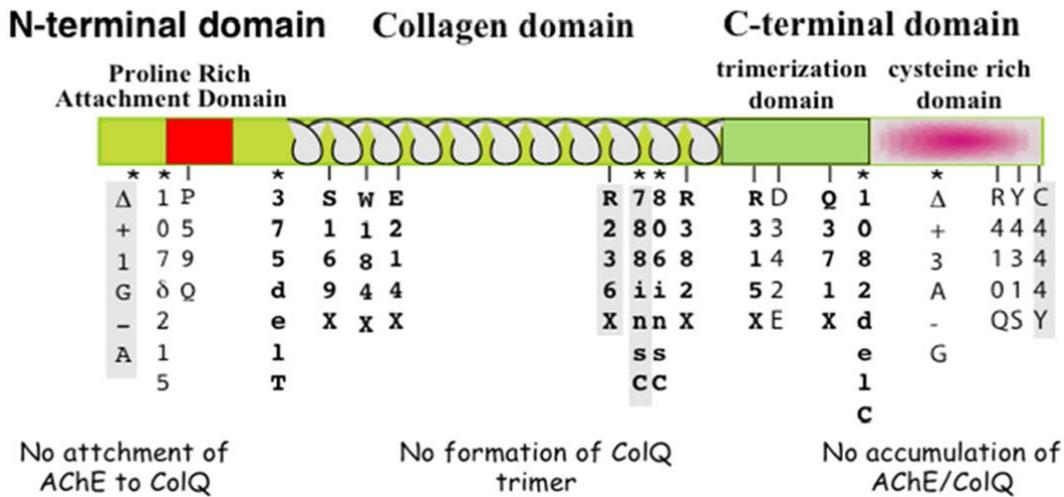


Figure 14. Schematic representation of ColQ and its gene mutations leading to CMS with AChE deficiency

A. ColQ is expressed from a single gene whose primary sequence is comprised of an N-terminal peptide region, a central collagen domain, and a C-terminal peptide. Through the interaction of their trimerization domain, the Collagen domain of each ColQ monomer associate to form a triple helix. A tetramer of AChE binds each branch of the triple helix. The polyproline rich domain (PRAD) of ColQ allows the covalent interaction with the C-terminus of AChE catalytic subunit. The two heparin binding site mediate ColQ's interaction with perlecan.

B. Currently known mutations in COLQ found in patients with CMS with AChE deficiency. ColQ is the most commonly mutated gene in patients with CMS with AChE deficiency.

V. HUR, A UBIQUITOUS POST-TRANSCRIPTIONAL REGULATOR

A. Post-transcriptional regulation in muscle

The post-transcriptional control of mRNAs begins as soon as the transcript emerges from the transcriptional machinery and is dependent on trans-acting factors such as microRNA and RNA binding proteins (RBPs) binding to their respective cis-elements in the target mRNA. These dynamic interactions allow for the proper control of RNA regulatory events, including RNA maturation, nucleocytoplasmic translocation, subcellular localization, degradation and translation. (Apponi et al., 2011).

1. RNA binding proteins in muscle

RBP form dynamic interactions with mRNA forming mRNA-bound ribonucleoprotein complexes (mRNPs). With more than 1100 genes involved in RNA recognition, but only 40 different types of domains involved in RNA recognition, the functional diversity of RNPs is dependent on the unique structural arrangement of multiple RNA binding domains (Finn et al., 2014; McKee and Silver, 2007).

RBPs play an important role in regulating key proteins involved in myogenesis. This section will discuss the currently known RBPs involved in myogenesis and at the NMJ.

a. HuR

The human antigen R (HuR) of the elav1 family of proteins is a ubiquitously expressed RBP which is present in the cytoplasm of differentiating muscle cells. HuR contains 3 RNA recognition motifs (RRM) that mediate binding to the U-rich and AU rich (ARE) cis elements on their target mRNA. Through the interaction with its cis-elements, HuR promotes cell cycle withdrawal of proliferating myoblasts and the differentiation of myoblast into myotubes.

HuR induces cell cycle withdrawal by altering its interaction with target transcripts. For example, in proliferating myoblasts, HuR forms a complex with the cell cycle control factor Cyclin D (Lal et al., 2004). This complex is stabilized by the Paired-like homeodomain transcription factor 2 (Pitx2). When differentiation is induced, Pitx2 is phosphorylated resulting in the destabilization and dissociation of the HuR/Cyclin D1/Pitx2 complex. This in turn destabilizes the Cyclin D1 transcript promoting cell cycle arrest (Gherzi et al., 2010). Inversely, the increase in HuR's interaction with cyclin dependent kinase inhibitor p21 stabilizes the transcripts and promotes cell cycle withdrawal (van der Giessen et al., 2003).

HuR stimulates myogenic differentiation by stabilizing myogenic factors MyoD and myogenin mRNA resulting in an increase in their transcript and protein levels. *In vitro* studies have shown that the knock-down of HuR inhibits myogenic differentiation (von Roretz et al., 2011).

b. KSRP

The KH-type splicing regulatory protein (KSRP) is a member of the far upstream element (FUSE)-binding protein family (FBP). KSRP interacts with AREs and stimulates 3'-5' exonucleolytic degradation of its target transcript (Briata et al., 2011). Like HuR, KSRP regulates p21, MyoD and myogenin transcript levels (Briata et al., 2005).

HuR and KSRP inversely regulate transcript stability to regulate myogenesis. During myoblast proliferation, KSRP destabilizes such transcripts as MyoD, myogenin and p21 inhibiting differentiation. However, when differentiation is induced, KSRP is phosphorylated inhibiting its interaction with ARE containing transcripts, allowing HuR to stabilize these transcripts promoting differentiation (Briata et al., 2005).

KSRP has also been focused on as a therapeutic approach in Duchenne Muscular Dystrophy (DMD). DMD is caused by mutations/deletions of the *Dystrophin* gene resulting in fiber degeneration and the invasion of adipos and connective tissues within muscle. Due to its high sequence identity and functional similarity with dystrophin, the upregulation of Utrophin A has been a therapeutic target for DMD (Amirouche et al., 2013a).

KSRP interacts with and destabilize Utrophin A mRNA in skeletal muscle. However, activation of p38 inhibits the destabilizing effect of KSRP on Utrophin A mRNA resulting in an increase in Utrophin A protein levels. Furthermore, a recent study has shown that the over expression of the skeletal muscle specific mir-206 increases Utrophin A mRNA levels. When mi206 is over expressed, it binds the 3'UTR of the KSRP transcript and down-regulates its expression. As a result, Utropin A levels are increased due to the liberation of its transcript from the destabilizing effects of KSRP (Amirouche et al., 2013b).

c. Staufen

Staufen is a double stranded RNA binding protein which regulates mRNA translocation, splicing, and degradation. Staufen-mediated mRNA decay (SMD) is dependent on Staufen binding to its cis-regulatory element located in the 3'UTR of its target transcripts. Once bound, Staufen promotes mRNA degradation by directly interacting with helicase activity (Park and Maquat, 2013).

In mammals, 2 homologues of staufen have been identified, Staufen 1 (Stau1) and Staufen2 (Stau2), both of which are expressed in skeletal muscle and have been shown to accumulate at the post-synaptic sarcoplasm of NMJs (Belanger et al., 2003; Buchner et al., 1999; Marion et al., 1999; Wickham et al., 1999). Stau1 and Stau2 expression have been found to be upregulated in myogenic differentiation and in cultured myotubes treated with the neuronal

factors agrin and neuregulin. These results indicate that Stau1 and Stau2 may be implicated in myogenesis and in the formation or maintenance of the NMJ (Belanger et al., 2003).

Stau1 proteins have been found to be increased in skeletal muscle of patients with Myotonic Dystrophy type 1 (DM1). DM1 is caused by the transcription of CUG repeats in the 3'UTR of the dystrophin myotonia protein kinase (DMPK) mRNA, which causes its localization in the nucleus and sequestration of transcription factors and RBPs. The misregulation of these proteins results in aberrant splicing, leading to the various symptoms of the disorder. Interestingly, Stau1 has been shown to bind the mutated DMPK transcript and induce its nuclear export and translation. Furthermore, Stau1 overexpression is capable of rescuing the alternative splicing of the aberrantly spliced mRNA in DM1. Thus the Stau1 RBP appears to play a positive role in the DM1 pathology (Ravel-Chapuis et al., 2012).

d. CUGBP1

CUG-binding protein 1 is part of the CUG-BP and ETR-3-like factors (CELF) family of RBPs. CUGBP1 regulates transcript stability and translation through the binding of its 3 RRM to multiple cis-elements including UG-rich elements, GC-rich elements, GU-rich elements (GREs) and AREs (Barreau et al., 2006; Vlasova et al., 2008).

CUGBP1 regulates multiple levels of myogenesis. CUGBP1 inhibits myogenesis by increasing the translation of MEF2A transcripts (Timchenko et al., 2004). Once myogenesis is induced, CUGBP1 promotes proliferation and differentiation by increasing the translation of Cyclin D1 and p21 respectively (Salisbury et al., 2008).

e. LIN-28

Lin-28 is cytoplasmic RNA binding protein that contains 3 different RNA-binding motifs: one cold shock domain (CSD) and two CCHC zinc finger motif. In mammals, Lin-28 is involved in the regulation of the growth and differentiation of embryonic tissue and stem cells and is down regulated once cellular differentiation is activated. However, Lin-28 expression is increased both *in vitro* during myoblast differentiation and *in vivo* in skeletal muscle regeneration and has been found to promote the translation of mRNA encoding for MyoD and IGF2 *in vitro* (Apponi et al., 2011; Polesskaya et al., 2007; Yang and Moss, 2003). The mechanism by which Lin-28 interacts with its target transcript is still unclear but appears to increase the stability and translation of its target mRNA through the interaction with single stranded uridine-rich elements flanked by guanosines (Hafner et al., 2013).

f. TTP

Tristetraprolin (TTP) is a zinc finger protein member of the TIS11 family of RBPs that interacts with AREs and destabilizes its target transcript through deadenylation mediated degradation (Hau et al., 2007; Lai et al., 1999; Lykke-Andersen and Wagner, 2005). TTP is activated in satellite cells following muscle injury however its target transcripts have yet to be identified (Sachidanandan et al., 2002).

B. The Hu/Elav Family

Hu/ELAV proteins are a mammalian family of RNA binding proteins orthologous to the embryonic lethal abnormal vision (elav) genes (Bell et al., 1988; Robinow and White, 1988; Szabo et al., 1991) and is named after the patient in which the Hu antibody was identified (Graus et al., 1997; Musunuru and Darnell, 2001).

The Hu/ELAV family contains 4 members, three of which are neuronal and include HuB/ELAV12, HuC/ELAV13, and HuD/ELAV14, and are implicated in neuronal

development, neuronal plasticity, and memory (Pascale et al., 2008). HuR (HuA/ ELAV11) was the last member of the Hu family to be cloned and characterized. HuR is highly conserved among vertebrates, with the human protein being 99.7% identical to that of mouse, 98.2% to chicken, and 90% to *Xenopus* (Fan and Steitz, 1998).

Unlike its family members, HuR is ubiquitously expressed in all cell types (Ma et al., 1997). HuR is embryonically lethal in mouse between embryonic days 10.5 and 14.5. HuR lethality results from its dominant role in organizing gene expression, guiding placental labyrinth morphogenesis, skeletal specification, and splenic ontogeny (Katsanou et al., 2009). HuR and its family members contain 3 RNA recognition motifs with high affinity for U- and AU-rich sequences (AREs), affecting their stability and/or translation (Ma et al., 1997).

C. HuR Function

1. mRNA degradation pathways

The post-transcriptional regulation of mRNA alters its translational efficiency and transcript stability, with vertebrates mRNA half-life ranging between 20min and 24hrs. RNA binding proteins (RBP) and noncoding RNA (microRNA) regulate mRNA half-life through the recruitment or the inhibition of degradation machinery.

In mammals, there are two main degradations pathways with the most common and the one utilized by HuR being the deadenylation dependent mRNA degradation pathway. Deadenylases degrade the mRNA poly(A)-tail through the binding of the mRNA 5' CAP, which in turn activates the ribonuclease activity of the deadenylase and further removal of the poly(A)-tail. Moreover, other forms of deadenylases remove the poly(A)-tail through a complex mechanism that is dependent on the translational machinery. Once the poly(A)-tail has been removed, the mRNA is fully degraded by 5'-3' and 3'-5' exosomes (Wu and Brewer, 2012).

The less common degradation pathway is known as the deadenylation independent pathway or the endoribonucleolytic pathway. This pathway is quite complex involving endoribonucleases which target actively translating mRNA and associate with their polyribosomes. This association activates the endoribonuclease, which then goes on to cleave mRNAs at its respective cleavage site (Wu and Brewer, 2012).

2. HuR binds ARE cis-elements

RBPs interact with cis-elements in their target transcript to regulate mRNA localization, translation, and degradation. Specifically, HuR interacts with the cis element called the Adenosine and Uridine Rich Element (ARE). With an estimated 5-8% of the transcriptome containing AREs, they were first discovered to serve as an mRNA decay function in 1986. AREs found throughout a transcript, mediate degradation through the previously described deadenylation dependent mRNA degradation pathway (Brennan and Steitz, 2001).

As more ARE elements were discovered, they were classed into three main groups. The first group, Class I AREs, contain 1 to 3 copies of the hallmark pentamer AUUUA sequence and are embedded within a Uridine rich region. The second group, Class II AREs, contain a minimum of 2 overlapping UUAUUUA(U/A)(U/A) sequences, again embedded in a Uridine rich region. Lastly, the third group, Class III AREs, do not contain the AUUUA pentamer but rather just the U-rich region, which may contain noncanonical AREs that have yet to be discovered (Chen and Shyu, 1995).

To date, twenty RBPs have been found to interact with AREs to induce mRNA degradation (ex. AUF1; KH-domain-splicing regulatory protein, KSRP), translation (ex. T-cell intracellular antigen-1, TIA-1), and mRNA stability (ex. HuR). It has been demonstrated that HuR stabilises its target transcript through the competitive binding with the destabilizing proteins (Zou et al 2010; Chang et al 2010; Fehir et al 2005; Linker et al 2005; Pautz et al

2006 and 2009). Furthermore, recent findings have found HuR acting together or in competition with microRNA towards the respective mRNA (Srikantan et al., 2012).

D. HuR Expression

1. Transcriptional regulation of HuR

Little is known about the regulation of HuR gene expression, but what is known is that HuR transcription is positively regulated by the transcription factor NF- κ B and by SMADS (Jeyaraj et al., 2010; Kang et al., 2008). However, the abundance of HuR mRNA and HuR protein are subject to multiple regulatory mechanisms. An overview of HuR expression and functional regulation is shown in figure 12.

2. The post-transcriptional regulation of HuR

a. Autoregulation

The HuR mRNA contains multiple alternative polyadenylation signals producing several polyadenylation variants varying in length. One of these longer variants contains a distal ARE sequence capable of binding HuR RBPs. Thus, HuR is capable of autoregulating itself through its self-binding, resulting in enhanced mRNA stability and enhanced mRNA cytoplasmic export (Al-Ahmadi et al., 2009).

b. MicroRNA

MicroRNAs are a class of noncoding RNAs whose final product is a 20-23 nucleotide functional RNA that controls gene expression. Specifically, microRNA target mRNA and either repress their translation or decrease their stability. Two microRNAs have been shown to interact with HuR mRNA. mir-519 interacts with two binding sites in HuR mRNA, one in the coding region and the other in its 3'UTR (Abdelmohsen et al., 2008). However, only the interaction of mir-519 to its site in the coding region functionally inhibits HuR translation.

Mir125a is the second microRNA known to associate with the 3'UTR of HuR mRNA and inhibit its translation (Guo et al., 2009).

3. Post-translational modifications of HuR regulate its localization

a. HuR shuttling

HuR is predominantly located in the nucleus in unstimulated cells, but is rapidly translocated to the cytoplasm upon various modifications induced by specific stimuli such as stress, apoptosis, cell division, and myogenesis (von Roretz et al., 2011). Due to the high level of HuR in the nucleus it is difficult to quantify the percentage of HuR that is translocated to the cytoplasm. However in an undifferentiated myoblast, there is about 10-fold more HuR in the nucleus than in other cellular compartments (Figueroa et al., 2003).

The shuttling of molecules between the nucleus and cytoplasm occurs through the nuclear pore complexes (NPC). Proteins and RNAs are carried through the NPC by way of transport receptors that recognize specific signals on their cargo molecules. The precise mechanism by which HuR is shuttled across the nuclear envelope is still unclear but requires the presence of its nucleocytoplasmic shuttling domain (HNS). Transport proteins, transportin 1 and 2 appear to serve a redundant role in HuR shuttling through their interaction with its HNS. Transportin 1 and 2 interact with HuR proteins and together interact with adaptor proteins pp32 or APRIL and are shuttled with the help of the CRM1 receptor (Guttinger et al., 2004; Rebane, 2004).

b. Post-translational modifications regulate HuR localization and function

The phosphorylation and methylation state of HuR plays a large role in HuR localization. The post-translational modification of HuR alters not only the subcellular localization of HuR but also its interaction with target mRNAs. Modifications in the RNA recognition motif affect the HuR binding affinity to target mRNAs, and modifications within or near the HuR Nucleocytoplasmic Shuttling sequence (HNS) alter HuR subcellular localization (Srikantan

et. al., 2012). The following is a list of known HuR modifications, with localization of modifications with respect to the RRM and HNS found in figure 15:

- i. **Checkpoint kinase Chk2** phosphorylates HuR at residues Ser-88, Ser-100, and Thr-118 and modulates its affinity to target mRNA (Abdelmohsen et al., 2008).
- ii. **Cdk1 (cdc25)** phosphorylates HuR at Ser-202 and induces 14-3-3 θ interaction with HuR resulting in its nuclear retention (Kim et al., 2008a).
- iii. **PKC family of kinases.** PKC α and PKC δ promote cytoplasmic export by both phosphorylating residue Ser-211 and HuR binding activity and by phosphorylating Ser-158 and Ser-318, respectively. PKC β alters HuR binding to its target mRNA but the residue in which it phosphorylates is still unknown (Doller et al., 2008; Doller et al., 2010).
- iv. **p38 (MAPK)** Induces HuR translocation to the cytoplasm by phosphorylating HuR residue Thr-118 (Lafarga et al., 2009).
- v. **Coactivator-associated Arginine methyltransferase 1 (CARM1)** is the only known methylator of HuR. CARM1 affects HuR target mRNA through the methylation of Arg-217 (Li et al., 2002).

Lastly, the cleavage of HuR has been shown to regulate HuR shuttling. In cells undergoing myogenesis or apoptosis, caspase-3/7-mediated cleavage of HuR is activated and HuR is cleaved at residue Asp-226. This generates a large 24kDa HuR cleavage product (HuR-CP1) and a smaller 8 kDa cleavage product, HuR-CP2. During myogenesis, for example HuR-CP1 binds transportin 2 and blocks the nuclear import of HuR and thus increase its cytoplasmic accumulation (Beauchamp et al., 2010).

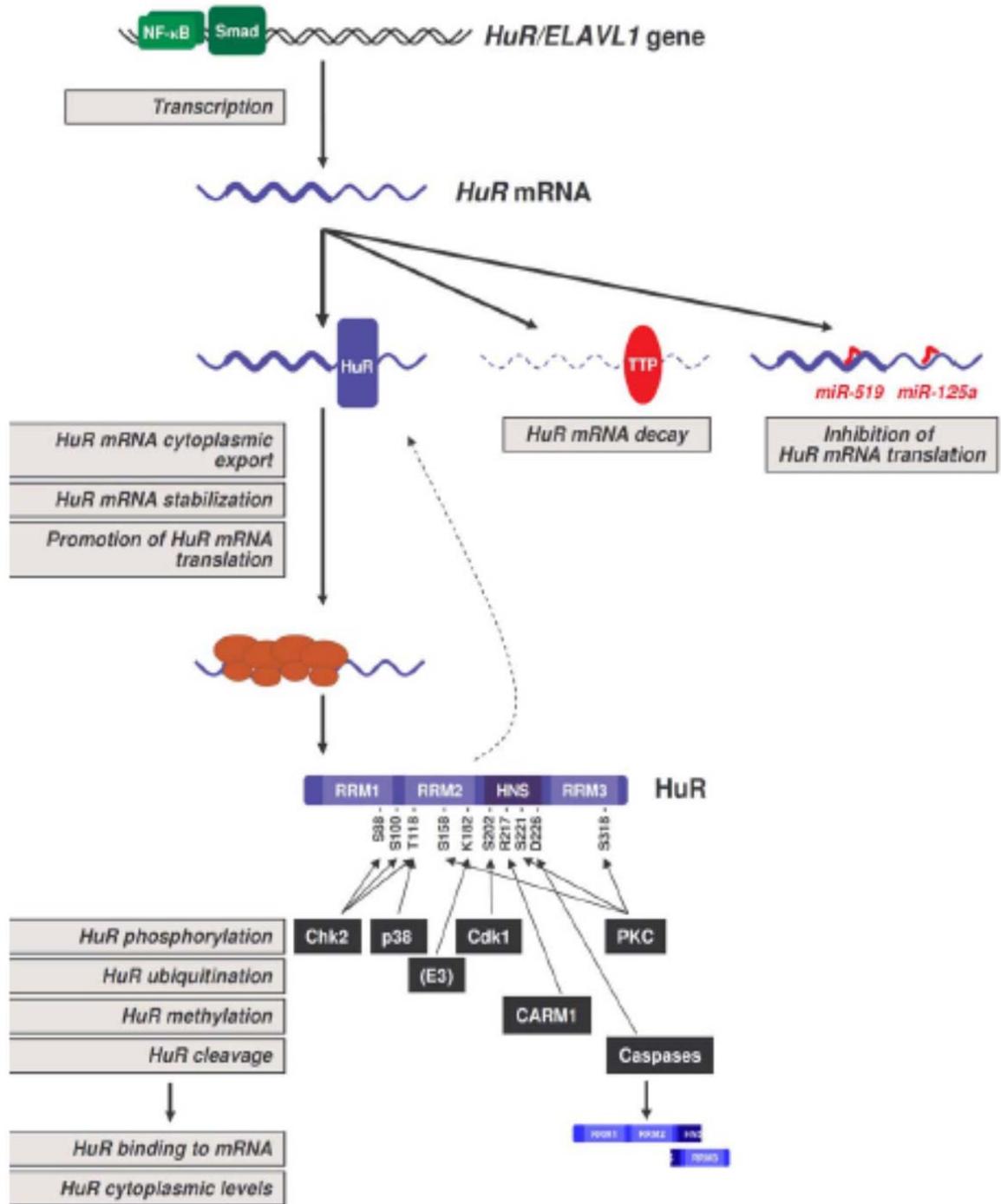


Figure 15. Regulation of HuR expression, localization and function.

The schematic depicts the current understanding of HuR regulation. Transcription of the HuR/ELAVL1 gene is controlled by the transcription factor NF- κ B. The HuR mRNA is positively regulated by enhanced export to the cytoplasm, stabilization, and enhanced translation but HuR itself; the HuR mRNA is negatively regulated by TTR-RBP tristetrarolin (TTP), which promotes HuR mRNA decay, and by microRNAs miR-125a and miR-519, which repress HuR translation. HuR protein is subject to phosphorylation by Chk2, which affects [HuR-mRNA] interactions, by Cdk1, which affects HuR levels in the cytoplasm, and by p38 and PKC, which affect both [HuR-mRNA] interactions and cytoplasmic HuR levels. Methylation by CARM1 can also affect HuR subcellular distribution and binding to mRNAs. Ubiquitination of HuR by an as-yet unknown E3 ligase controls HuR protein stability, and caspases can cleave HuR into two fragments with different cellular properties. Gray squares indicate steps in which HuR expression or function are regulated (Srikantan and Gorospe, 2012).

E. HuR at the NMJ

1. HuR stabilises key players of the NMJ

Previous studies have reported that Human antigen R (HuR) a member of the ELAV/Hu family of RBPs is critical during skeletal myogenesis and post-synaptic NMJ formation. HuR has been shown to coordinate the regulation of muscle early and late onset differentiation genes including myogenin, MyoD and AChE (Deschenes-Furry et al., 2005; Figueroa et al., 2003). At the level of the motor neuron, HuR is particularly implicated in Schwann cell development, regulating a vast number of genes required to promote proliferation, apoptosis, and morphogenesis (Iruarrizaga-Lejarreta et al., 2012).

2. HuR is a therapeutic target of NMJ diseases

Spinal muscle atrophy (SMA) is a disease characterized by the progressive loss of α motor neurons and consequently muscle atrophy, essentially affecting proximal muscle. One of the hallmarks in this disease is the disruption of the NMJ (Kariya et al., 2008; Pearn, 1978). SMA is caused by the homozygous deletion of the survival motor neuron gene (*SMN*) (Lefebvre et al., 1995). In Humans, two homologous copies of the *SMN* genes exist, the telomeric *SMN* gene copy (*SMN1*) and the centromeric *SMN* gene copy (*SMN2*) with 95% of SMA patients lacking the *SMN1* gene. The *SMN2* gene is unable to compensate for the loss of *SMN1* because only 5-10% of *SMN2* transcripts form the full-length functional *SMN* transcript. The majority of *SMN2* transcripts undergo alternative splicing of its exon 7 resulting in a truncated transcript and non-functional *SMN* proteins (Lefebvre et al., 1995; Lorson et al., 1999; Monani et al., 1999).

Recently, it has been shown that *SMN* mRNAs are stabilized by HuR and that p38 activation increased levels of *SMN* proteins in this fashion (Farooq et al., 2009). Thus, inducing HuR mediated stabilization of the *SMN* mRNA increases the percentage of functional *SMN*

protein. In support of these findings, *in vitro* and *in vivo* treatment of Celecoxib, an activator of p38, has been shown to increase the levels of SMN proteins by activating the p38 pathway (Farooq et al., 2013). Furthermore, celecoxib treatment improved motor function and increased survival of SMA mouse models, thus highlighting the importance of HuR as a therapeutic target for muscular diseases.

VI. OBJECTIVES OF THE THESIS – DECIPHERING ColQ INDUCED MECHANISMS IN THE CONTROL OF AChR mRNA LEVELS

ColQ is a secreted protein which anchors AChE to the post synaptic membrane of the (NMJ) via its interaction with the muscle specific kinase (MuSK) and perlecan (Cartaud et al., 2004; Peng et al., 1999). To date, over 30 mutations in ColQ have been linked to congenital myasthenic syndromes with AChE deficiency, with a number of these mutations in the C-terminal region, which binds MuSK a vital protein in NMJ formation and maturation. Our group has opened a new door into the function of ColQ as not just an anchoring protein, but also as a signalling molecule in the muscle. We have demonstrated that through its interaction with MuSK, ColQ regulates the expression of post-synaptic proteins, one of these being the AChRs, which are clustered at the NMJ (Sigoillot et. al., 2010).

The critical role of ColQ at the mammalian NMJ is thought to be mediated through the accumulation of AChE in the synaptic basal lamina (Krejci et al., 1999; Krejci et al., 1997). Indeed, AChE restricts, in space and time, the function of ACh, by hydrolyzing this neurotransmitter in the synaptic cleft. However, we now want to further analyse the critical role of ColQ as a signalling molecule at the NMJ and how ColQ's new role is linked to the symptoms of CMS with AChE deficiency. To gain insight into the signalling role of ColQ, I formulated three objectives:

A. Identifying ColQ's Phenotypic and Molecular Effects in a Model of CMS With AChE Deficiency.

The putative signalling role of ColQ raised by its interaction with MuSK intrigued us to determine the molecular and phenotypic hallmarks of CMS with AChE deficiency in a mouse model and explore the role of ColQ as more than an anchoring protein. By invalidating the ColQ gene, a mouse model of CMS has been generated in the lab of J. Massoulié in

collaboration with the group of J Sanes. Using the WT and ColQ knock-out mice, immortalized WT and ColQ deficient skeletal muscle cell lines were created.

We conducted a large genetic screen to identify the molecular targets of ColQ *in vitro* and revealed molecular defects that could explain the observed phenotype in the mouse model of CMS with AChE deficiency. These studies show that under-expressed genes in the absence of ColQ belong primarily to the extracellular matrix. In the basal lamina, ColQ interacts with perlecan, which itself interacts with dystroglycan creating an indirect link to the dystrophin complex. We demonstrate that in the absence of ColQ, mRNA levels of both perlecan and dystroglycan are increased *in vitro*, although only dystroglycan is increased *in vivo*. The increase in dystroglycan mRNA levels has sparked our interest since it connects the ECM to the intracellular cytoskeleton of the muscle. Morphological studies of muscles in wild type and ColQ-deficient mice at various stages of post-natal development have also been performed. However, the most striking molecular consequence of ColQ deficiency is the upregulation of AChR mRNA *in vitro* and *in vivo*. Thus, my main following goal was to define the cascade linking ColQ to AChR mRNA levels. This objective is discussed in Annex A.

B. Deciphering the Mechanism by Which ColQ Regulates AChR Subunit mRNA at the NMJ.

The absence of ColQ increases all five AChR subunit mRNAs and thus, I explored the cascade linking this extracellular collagen to the muscle metabolism of mRNA. One of the mechanisms regulating mRNA levels is via the regulation of their stability, by trans-acting factors such as RBPs or microRNAs. Previous studies have reported that HuR, a member of the ELAV/Hu family of RBPs, is critical during skeletal myogenesis and its post-synaptic NMJ formation. HuR has been demonstrated to coordinate the regulation of muscle, early and

late onset differentiation genes including myogenin, MyoD and AChE (Deschenes-Furry et al., 2005; Figueroa et al., 2003). Therefore, I investigated the possibility that ColQ could control AChR mRNA stability through a mechanism mediated by HuR.

In this study, we provide insight into the mechanism that regulates ColQ mediated AChR subunit expression. Since the AChR β subunit is essential for AChR clustering, we focused on the metabolism of β subunit mRNA. We found (1) that the absence of ColQ increases AChR β subunit mRNA stability, (2) using RNA immunoprecipitation, that HuR interacts specifically with the ARE in the AChR β 3'UTR to positively regulate AChR β subunit expression and (3) that ColQ controls HuR levels at least partially by p38 activation.

C. Providing an Updated and Integrative View of ColQ's Functions.

A little under 40 years has passed since the first hint of a collagen tail was found to be associated with AChE. In this review, I provide a brief summary of ColQ since its cloning in 1993 as an anchoring protein to AChE in the synaptic cleft of the NMJ. I then proceed to present the latest results on ColQ function beyond that of an anchoring protein and uncover the current state of ColQ as a signalling molecule. I will discuss ColQ's regulation of AChR clustering and MuSK insertion at the NMJ post-synaptic membrane along with the expression of a number of specific synaptic genes.

The results obtained for each of the first two objectives are included in the two articles which are currently submitted (Article 2) and in preparation (Article 1). A review article, published in *Chemical Biological Interactions* (2013), corresponds to the third objective.

RESULTS

I. DECIPHERING THE MECHANISM BY WHICH ColQ REGULATES AChR SUBUNIT mRNA AT NMJ.

A. Introduction

C. Legay's team in collaboration with the group of J. Cartaud, has shown that ColQ interacts with a muscle specific tyrosine kinase called MuSK (Cartaud et. al., 2004) that controls the clustering of AChR. The team further demonstrated that the expression of a number of specific synaptic genes is affected (Sigoillot et al., J Neurosci., 2010, and Sigoillot et al., Chem Biol Interact., 2010). More specifically, Sigoillot et al showed that ColQ deficiency leads to increased levels of all five AChR subunit mRNAs *in vivo* and *in vitro*. Although the upregulation of AChR and rapsyn mRNA can be correlated to a higher density of AChR clusters. However, these clusters are smaller due to the under expression of MuSK at the membrane. This abnormal level of membrane bound MuSK is accompanied by a decrease in MuSK signaling. Indeed, agrin has been shown to induce the phosphorylation of the AChR β subunit via rapsyn, an effect which is decreased in ColQ deficient muscle (soleus and sternomastoid)

Sigoillot et al. also found an increase in MuSK and rapsyn mRNA levels in the absence of ColQ *in vitro*, however only rapsyn mRNA levels were increased *in vivo*. ColQ knock-out mice were found to have a lower concentration of membrane bound MuSK associated with AChR clusters but a higher concentration of rapsyn associated AChR clusters (Sigoillot et al., 2010a).

Since AChR γ and ϵ subunit mRNA were both upregulated *in vivo* in the absence of ColQ at P30, it raises the question of the structure of the AChR pentamer in ColQ-deficient mice. On going experiments using immunohistochemistry show that mixed AChR incorporating γ or ϵ subunits are present at the NMJ. Since γ and ϵ AChR have different kinetics of open channel

time, this last result could impact the understanding of the pathophysiology of the NMJ in CMS with AChE deficiency.

The proper functioning of the NMJ is dependent on AChR, with post-synaptic development and maintenance being focalized on AChR expression and localization. As a result, we sought out to decipher the mechanism by which ColQ regulates AChR subunit mRNA. Likewise, because AChR β was shown to be significantly effected *in vitro* and *in vivo* by ColQ and because it plays an important role in the postsynaptic localization of the receptor at the developing synapse, we focused our study on this subunit.

One of the mechanisms regulating mRNA levels is through the regulation of their stability by trans acting factors such as RNA binding proteins (RBPs) or microRNAs. Previous studies have reported that Human antigen R (HuR) a member of the ELAV/Hu family of RBPs is critical during skeletal myogenesis and its post-synaptic NMJ formation both *in vitro* and *in vivo* (Figueroa et al., 2003). HuR has been known to coordinate the regulation of muscle early and late onset differentiation genes including myogenin, MyoD and AChE (Deschenes-Furry et al., 2005; Figueroa et al., 2003). Therefore, to further investigate the mechanism behind the increase in AChR mRNA, I have collaborated with the Laboratory of Dr. Bernard Jasmin at the University of Ottawa. Dr. Jasmin has focused a considerable amount of efforts to study the post-transcriptional events regulating expression of specific mRNA in skeletal muscle.

B. Primary Results

The most striking effect of the absence of ColQ is an upregulation of all AChR subunit mRNAs correlated by an increase in their protein levels (Sigoillot et al., 2010a). Here, we show that the upregulation of AChR β mRNAs is induced by a post-transcriptional mechanism. *In vitro* mRNA stability assays using Actinomycin D were performed in a WT and ColQ-deficient muscle cell lines (MLCL) produced by my lab. We chose our MLCL cell line over the C2C12 cell line because unlike the C2C12, our WT cell line forms the characteristic AChE clusters suggesting that this cell line differentiates better than the C2C12 (Sigoillot et al., 2010b). Actinomycin D inhibits transcription allowing for the comparison of mRNA decay in the WT and ColQ deficient myotubes. When comparing the two cell lines, AChR β subunit mRNA degradation was significantly lower in the absence of ColQ when compared to WT (Article 1, Fig.1). This indicates that the absence of ColQ increased AChR mRNA stability which correlates with the previously observed increase in AChR mRNA levels.

The above results lead us to determine the identity of trans-acting factors that can influence the ColQ dependent stability of AChR subunit mRNA. In this context, it has been shown that the ubiquitously expressed HuR protein plays a critical role in differentiating muscle cells by interacting with the AU rich element (ARE) contained in the 3'UTR of target mRNA leading to their stabilization (Figuroa et al., 2003). Bioinformatics analysis using the online data base AREsite to identify classic ARE sites (Gruber et al., 2011), found that AChR β and two other subunits (γ and δ) contain at least one computationally predicted HuR site in their 3'UTR. Furthermore, HuR protein quantification showed a similar result in the absence of ColQ, resulting in a significant increase in HuR in two of the three stages of myotube differentiation, with a 40% increase at T1 and a 60% increase five days after differentiation was induced (Article 1 Fig.2).

Using RNA-IP followed by reverse transcription-QPCR we tested the binding of HuR to its putative ARE site within the AChR β . We first tested the endogenous interaction in myogenic cells, which showed that HuR indeed interacted with AChR β subunit mRNA (Article 1, Fig. 3). Furthermore, the interaction site of HuR in the 3'UTR of AChR β was analysed by transfecting luciferase reporter constructs containing the full-length 3'-UTR of AChR β in COS cells expressing endogenous HuR.

RNA-IP experiments done on the lysates from the transfected COS cells demonstrated that HuR specifically interacts with the ARE sequence located in the 3'UTR of the β subunit, as this interaction was lost when COS cells were transfected with a luciferase reporter construct containing the full-length 3'-UTR of AChR β with a mutated ARE site (Article 1, Fig. 4A & B).

To assay for the role of HuR after its recruitment to the ARE element in the 3'UTR of AChR β , COS cells were transfected with WT or ARE mutated reporter constructs. The interaction of HuR with the reporter construct transcript resulted in a decrease in transcript levels of 37% (Article 1, Fig. 4C). Furthermore, the overexpression of HuR was found to increase WT reporter mRNA levels by 200% when compared to pcDNA3 vector control. The mutant reporter construct on the other hand did not alter its mRNA levels in the presence of HuR overexpression (Article 1, Fig.4D, E). Lastly, Actinomycin D stability assays showed that the HuR dependent increase in luciferase mRNA was caused by an increase in its stability (Article 1, Fig.4F).

The p38 MAPK pathway plays an important role in post-transcriptional regulation observed in ARE containing mRNA, with p38 α specifically regulating the abundance and/or activity of HuR (Lafarga et al., 2009). The mammalian p38 MAPK pathway was initially recognised for its activation upon cellular stress. P38 has now been shown to also regulate cell survival and

differentiation. Four isoforms of p38 exist in mammals, α , β , γ , and δ , each of which differ in their expression patterns and substrate specificity (Cuenda and Rousseau, 2007). The canonical pathway of p38 activation involves the phosphorylation of its activation loop at its Thr-Gly-Tyr sequence by three MAPK-activated protein kinases/Mitogen-activated protein kinase kinases (MKKs/MAP2Ks): 1) MKK6 phosphorylates all four p38 isoforms 2) MKK3 phosphorylates p38 α , δ , γ and 3) MKK4 phosphorylates p38 α . The phosphorylation of the active loop by these kinases induces a conformational change, stabilizing the activation loop in an open conformation for substrate binding. Once phosphorylated, p38 goes on to phosphorylate its substrate on Ser-Pro or Thr-Pro motifs (Cuadrado and Nebreda, 2010).

In the muscle, three isoforms of p38 ($\alpha\beta\gamma$) are indispensable for differentiation (Wang et al., 2008). Thus, because HuR is regulated by p38 α , we set out to determine whether the absence of ColQ induces the activation of p38 α signaling at the NMJ by comparing the phosphorylation state of p38 α in WT and ColQ deficient myotubes. Western blotting demonstrated that ColQ deficient myotubes had a 274.4% increase in phosphorylated p38 when compared to that of the WT (Article 1, Fig.6A, B). Moreover, the inhibition of p38 α/β using SB203580 resulted in a 15% and 48% decrease in HuR protein levels in myotubes at T1 and T2 respectively (Article 1, Fig.6 C, D).

C. Conclusion and Perspectives

This study provides insight into a possible mechanism explaining the functional role of the synaptic collagen ColQ. Specifically, we have demonstrated that ColQ regulates the intracellular expression of AChR subunits through a mechanism requiring the RNA binding protein HuR. We have provided the first evidence, that in addition to transcriptional regulation, post-transcriptional events are also capable of modulating the expression of AChR

subunits. In the present study, it was found that AChR β mRNAs are a post-transcriptional target for the stabilizing effects of HuR.

After completing the study, five main questions still remain to be answered:

- 1- Could ColQ also affect the transcription of AChR subunit genes in parallel with the post-transcriptional mechanisms?
- 2- Could microRNA or other cofactors play a role in AChR mRNA stability?
- 3- Is the ColQ dependent upregulation of the remaining 4 AChR subunits also occurring through the HuR dependent mechanism?
- 4- Is p38 the primary pathway inducing the ColQ regulated HuR dependent increase in AChR subunit mRNA? What is the relationship between MuSK and p38? What are the stimuli upstream of p38?
- 5- Does HuR exert the same effect on the ColQ dependent regulation of AChR subunit mRNA *in vivo*?

1- Could ColQ also affect the transcription of AChR subunit genes in parallel with the post-transcriptional mechanisms?

Since AChR subunit mRNA upregulation is an important hallmark phenotype of ColQ deficiency, we wanted to determine if transcription played a role in the ColQ regulated AChR subunit mRNA levels. Synapse formation induces an increase in the levels of AChR and other post-synaptic proteins. As previously mentioned, AChR gene expression is thought to be induced by the mitogen-activated protein kinase (MAPK), c-jun NH₂-terminal kinase (JNK) and the Phosphatidyl Inositol-3 kinase (PI3K) signaling pathways. These pathways converge to phosphorylate and activate the Ets family transcription factor, GA binding protein (GABP), *in vitro*. Phosphorylated GABP binds an N-box (CCGGAA) in the promoters of synaptic genes inducing their transcription (Schaeffer et al., 2001; Schaeffer et al., 1998).

To determine if the absence of ColQ is increasing neuregulin dependent AChR transcription, we inserted either the WT promoter region of the AChR δ subunit (δ luci) (839 nt of the promoter; Koike et al., 1995) or a mutated AChR δ promoter with a deleted N box (δ luci Δ N), upstream of a luciferase reporter gene. These constructs were then co-transfected with the control vector β -galactosidase into WT and ColQ mutant cells and further induced to differentiate. Four days after differentiation, both cells lines were treated with NRG1 for 16hrs and further lysed.

Figure 16, shows that in the absence of NRG1 stimulation, the luciferase activity remains relatively unchanged in WT and ColQ deficient myotubes transfected with the δ luci vector. Furthermore, WT and ColQ deficient myotubes demonstrated a significant decrease in luciferase activity when transfected with δ luci Δ N, confirming that the N box plays a primary role in AChR transcription in both cell lines.

The treatment of NRG1 increases luciferase activity by 185% and 165% in WT and ColQ deficient myotubes respectively, both of which being transfected with δ luci. This increase is significantly lost in both cell lines expressing δ luci Δ N. Therefore, based on the quantification of the luciferase activity, which mimics the transcriptional activity of AChR δ , we were able to show that the transcriptional activity in WT and ColQ deficient cells of the δ subunit was not significantly different and it must be determined if a similar result is occurring with other subunits.

In support of this finding, microarray studies completed in my laboratory showed that factors known to regulate the transcription of AChR are not differentially expressed in WT versus ColQ deficient muscle cells. Thus, these two studies suggest that ColQ is not regulating AChR subunit mRNA at the transcriptional level.

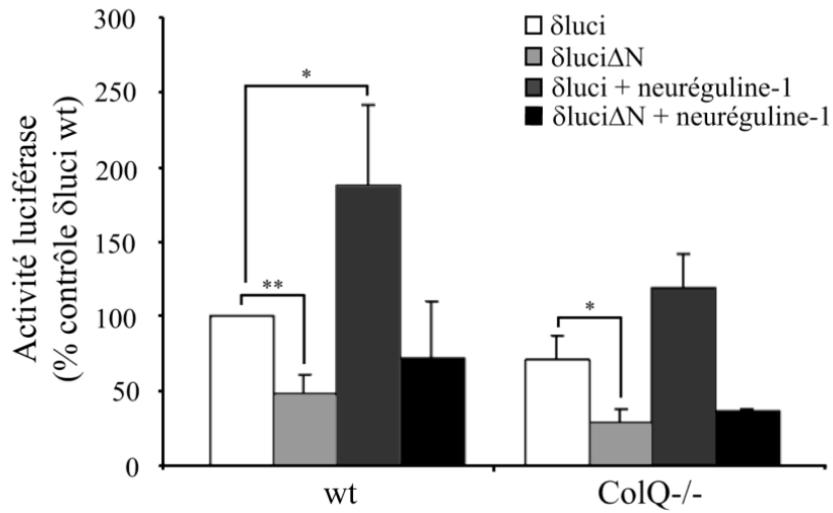


Figure 16. *The transcriptional activity of the AChR δ subunit in WT and ColQ^{-/-} myotubes at T2. WT and ColQ deficient muscle cells were transfected with the pSV- β -galactosidase control vector and the δ luciferase construct (Luciferase gene under the control of the AChR δ promoter) or δ luciferase Δ N (Luciferase gene under the control of the AChR δ promoter which has had its N box deleted). The transfected muscle cells were treated for 16h with neuregulin-1 (5nM). Luciferase activity was normalized to the transfection efficiency using β -galactosidase ($n \geq 3$).*

2- Could microRNA or other cofactors play a role in AChR mRNA stability?

Once mRNA is transcribed, its stability is regulated by various factors, one of which includes trans-acting factors which bind specific cis-elements in their target mRNA. Initially my interest was drawn to two types of trans-acting factors; the mRNA binding protein HuR and microRNA, mi1/206, both of which bind the 3'UTR of their target mRNA. MicroRNAs are a class of noncoding RNAs whose final product is a ~22 nucleotide functional RNA that controls gene expression. Specifically microRNA target mRNA and either repress their translation or decrease their stability.

Mir-1/206 is a member of the highly conserved muscle specific mir-1 family, one of the few microRNA present in the last common ancestor (McCarthy, 2008). Mir-206 is only expressed in skeletal muscle and is the most abundant microRNA in skeletal muscle. Mir-206 has a highly important role in myogenesis, regulating the expression of such genes as MyoD (McCarthy, 2008). Therefore, we wanted to determine if mir-206 could be a possible candidate for ColQ dependent increase in AChR mRNA levels.

Using the online database TargetScan, each AChR subunit was scanned for target microRNA sites conserved within their 3'UTR. Specifically, TargetScan searches for the presence of conserved 8mer and 7mer sites that match the seed region of the microRNA. Results from this scan did not show the presence of conserved binding sequences for microRNA1/206 in the 3'UTR of AChR subunit mRNAs. In fact, none of the muscle specific microRNA such as that of mir-133, which is specific to skeletal and cardiac muscle, contains conserved binding sites in any of the AChR subunits. Therefore, it appears that AChR may not be regulated by muscle specific microRNA. Still, because synaptic nuclei are capable of expressing neuronal genes, it is possible that the synaptic nuclei of muscle fibers may express neuronal specific microRNAs which are capable of interacting with the transcripts of the AChR subunit. Preliminary analysis, using the TargetScan software, shows that the AChR β subunit contains

a binding site for neuronal mir-124. The remaining subunits however do not appear to contain binding sites for the currently known neuronal microRNA.

The mRNAs coding for synaptic proteins MuSK, LRP4 and rapsyn are also upregulated in the absence of ColQ (Sigoillot et al., 2010a). When screening the 3'UTR sequences of MuSK, LRP4, and rapsyn, only LRP4 and MuSK mRNA were found to contained ARE sites (Sigoillot et al., 2010a). It should also be noted that MyoD and Myogenin, which have been shown to bind HuR (Figuroa et al., 2003), are not upregulated in the absence of ColQ at the time of differentiation explored (Sigoillot et al., submitted). AChE mRNAs have also been identified as targets of HuR (Deschenes-Furry et al., 2005), however their increase is only significant at one time-point of myotube differentiation *in vitro* (S. Sigoillot, F. Bourgeois, J. Karmouch, E. Krejci, C. Chevalier, R. Houlgatte, J. Leger, and C. Legay, in preparation). HuR is capable of acting together or in competition with other RBPs and microRNAs through the competitive or co-binding to the respective mRNA at its ARE. Thus it is possible that for these set of synaptic genes, HuR levels are not competitive enough to interfere with other RBPs or microRNAs (Srikantan et al., 2012).

Little is known concerning the competitive binding of HuR with other RBPs and microRNA, however it may involve their upregulation or down regulation during a given cellular context. For example, AChE is expressed both in the muscle and in the nerve. Interestingly, in the muscle, AChE transcripts are stabilized by HuR while in the nerve, another neuronal Hu/elav family member, HuD, targets the same ARE site and stabilizes AChE. In The neuron, AChE stability is dependent on HuD and mir-132 (Bronicki and Jasmin, 2012).

Furthermore, changes in levels of microRNA have been shown to alter its target transcript. For example, Utrophin A is post-transcriptionally down regulated by the RBP KSRP and the skeletal muscle specific microRNA mir-206. Interestingly, a rescent study has shown that the over expression of mir-206 increases Utrophin A mRNA levels. When mi206 is over

expressed, it binds the 3'UTR of the KSRP transcript and downregulates its expression. As a result, Utrobin A levels are increased due to the liberation of its transcript from the destabilizing effects of KSRP (Amirouche et al., 2013b). Therefore, in our case it may be that the absence of ColQ may render HuR levels competitive to other RBPs and/or microRNA, to stabilise transcripts of only a group of synaptic transcripts.

3- Is the ColQ dependent upregulation of the remaining 4 AChR subunits also occurring through the HuR dependent mechanism?

i. HuR Binds AChR Subunit mRNA in Muscle Cells

Previously we have shown that in the muscle cell lines generated in my lab, HuR regulates the ColQ dependent upregulation of AChR β subunits (Article 1). Since the levels of all AChR subunit mRNAs are increased in the absence of ColQ, we wondered if HuR could be used as a common mechanism to coordinate the upregulation of these mRNAs. Thus, we have begun testing the interaction between HuR and the α and γ subunit mRNA.

In order to test the endogenous binding of HuR to the AChR α and γ subunit mRNA, we performed RNA IP experiments with cultured myotubes. After formaldehyde cross-linking and HuR or IgG (negative control) immunoprecipitation, co-immunoprecipitated mRNAs were analyzed by quantitative RT-QPCR (Figure 17). Our results clearly show that in myogenic MLCL cell line, HuR indeed interacted with AChR α . Preliminary data indicates a similar result for the γ subunit and not with the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) that has no ARE sites.

The interaction between HuR and AChR α subunit is unexpected as there is no classical ARE binding site identified in its 3'UTR. The nicotinic AChR receptor ion channel is a pentamer composed of four subunits, two of which are α , 1 β , 1 δ , and 1 γ/ϵ . In addition to being the highest represented subunit in the AChR, the α subunits is also the “seeding” subunit in the two proposed mechanisms in the formation of the ion channel (Green, 1999, Dvir et al.,

2004). However, little is known concerning the regulation of α subunit expression, therefore it is possible that the α subunit contains non classical ARE sequences that have yet to be identified.

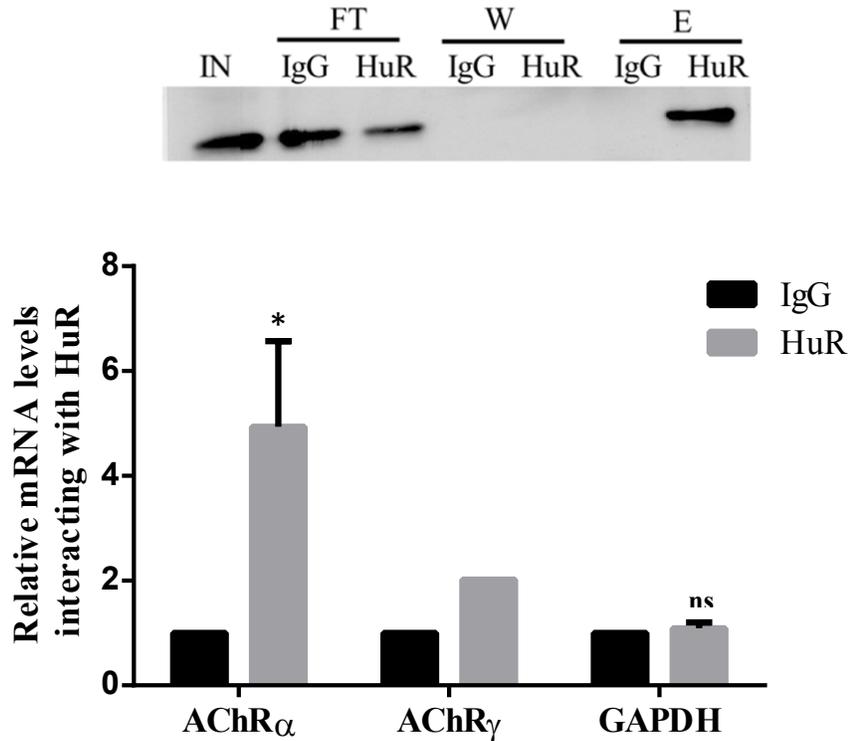


Figure 17. HuR binds endogenous AChR subunit mRNA in myotubes. A representative western blot confirming the quantity and precipitation of HuR is depicted (top panel; IN, input; FT, flow through; W, wash; E, Elution). Quantification of the interaction (bottom panel) shows that endogenous HuR interacts with AChR mRNA *in vitro*. In 5 day differentiated myotubes, RNA–protein complexes were crosslinked with formaldehyde and immunoprecipitated after cellular lysis, using antibodies against HuR or IgG. Following crosslink reversal, the RNA was isolated from the immunoprecipitate and was used to produce cDNA by reverse transcription, followed by QPCR quantification with AChR α and γ subunit primers. Data are presented as percentage of control SEM (AChR α n=5; AChR γ n=1; GAPDH n=3). * p <0.05; ns, not significant; unpaired Mann–Whitney’s U test.

To confirm that the above results are not specific to the MLCL cell lines, the association of HuR with endogenous AChR subunit mRNA was investigated in the C2C12 muscle cell line by RNA immunoprecipitation according to the same methodology used with the MLCL cell line. To assess whether endogenous HuR protein interacts with and potentially stabilizes endogenous AChR subunit transcript, RNA–protein complexes were cross-linked using formaldehyde in C2C12 myotubes. RNA–protein complexes were then immunoprecipitated with IgG or HuR antibody after cellular lysis. After cross-linking reversal, cDNA was reverse transcribed from RNA isolated from the immunoprecipitate, followed by QPCR amplification with AChR subunit primers. Preliminary results indicate that HuR may interact with AChR α and γ subunit and not with the GAPDH in C2C12 (Figure 18). In conclusion, regardless of the muscle cell line, it appears that HuR interacts with at least three of the five AChR subunit transcripts tested (α , β , γ).

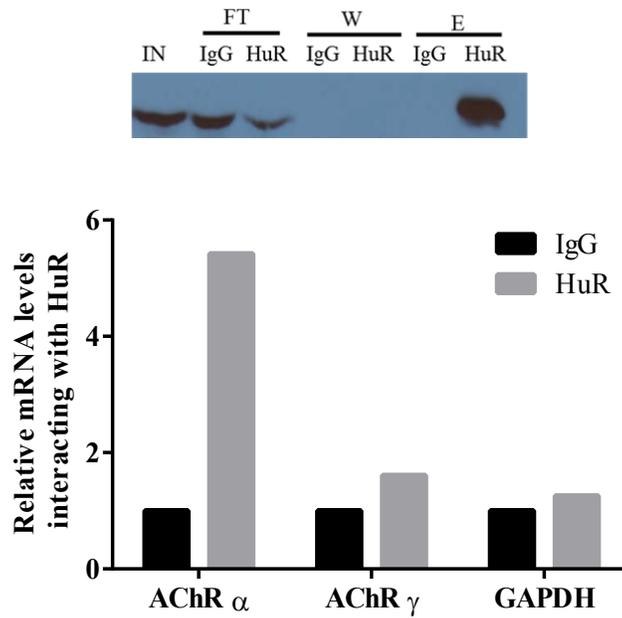


Figure 18. *HuR is capable of binding endogenous AChR subunit mRNA in C2C12 myotubes. A representative western blot confirming the quantity and precipitation of HuR is presented above (top panel; IN, input; FT, flow through; W, wash; E, Elution). Quantification of the interaction (bottom panel) shows that endogenous HuR interacts with AChR mRNA in vitro. In 4 day differentiated myotubes, RNA–protein complexes were crosslinked with formaldehyde and immunoprecipitated after cellular lysis, using antibodies against HuR or IgG. Following crosslink reversal, the RNA was isolated from the immunoprecipitate and was used to produce cDNA by reverse transcription, followed by QPCR quantification with AChR α and γ subunit primers ($n=1$).*

ii. The role of 3'UTR in AChR mRNA stabilization

We evaluated the contribution of the conserved ARE in the AChR β mRNA/ HuR interaction by cloning both a WT and mutated form of the full-length 3' UTR of the mouse AChR β subunit transcript and inserted it downstream of the luciferase reporter gene driven by a constitutive promoter (Article 1: Figure 4A). Using these constructs (produced by Guy Bélanger in the laboratory of Pr. Bernard Jasmin), we were able to conclude that HuR interacts with the conserved ARE element in the 3'UTR of AChR β subunit (Article 1: Figure 4B). We have used a similar approach to evaluate the contribution of the conserved ARE in the AChR α and γ / HuR interaction. We cloned both a WT and mutated form of the full-length 3' UTR of the mouse AChR γ subunit transcript and inserted it downstream of the luciferase reporter gene, driven by a constitutive promoter. The mutated clones contains two point mutations in the ARE element (identical to Article 1: see figure 4A), which is expected to prevent it from recruiting HuR.

The constructs were individually transfected into COS cells which already express endogenous HuR (Fan and Steitz, 1998). RNA IP analysis, completed 48hr after transfection, showed a significant interaction of the WT AChR γ reporter construct when compared to that of the empty vector (Figure 19). Mutation of the conserved ARE resulted in a significant 13 fold decrease in the binding of HuR to the AChR γ full length 3'UTR, with measured abundance of reporter mRNA in the immunoprecipitates being similar to that of the empty vector. Taken together, these results showed that HuR also interacts with the conserved ARE element in the 3'UTR of AChR γ subunit.

As AChR α subunit does not contain a conserved ARE element, we wanted to confirm that HuR is in fact interacting with 3'-UTR of AChR α mRNA. To this end, we engineered a luciferase reporter construct in which the endogenous 3'-UTR was replaced with the mouse AChR α subunit 3'-UTR. As AChR α subunit contains two polyadenylation signals, we chose

the first of the two signals as previous studies have demonstrated that in muscle, the first polyadenylation signal is used preferentially over the second signal (Boulter et al., 1985) . As a result, there is significantly more of the 2.0-kb AChR α mRNA in C2C12 cells than the 4.0-kb transcript corresponding preferentially to the choice of the first poly-adenylation signal over the choice of the second (Boulter et al., 1985). RNA IP analysis, completed 48hr after transfection, revealed that the luciferase α 3'UTR construct appeared to have a minute interaction with HuR (Figure 20). Although HuR binds the endogenous α subunit transcript. In conclusion, I have provided preliminary data demonstrating that HuR interacts with both AChR α and γ subunits. HuR's interaction with the γ subunit is in fact occurring through the ARE element located in its 3'UTR. However, the HuR interaction site for the α subunit is still unknown, but it appears to not be located in the 3'UTR.

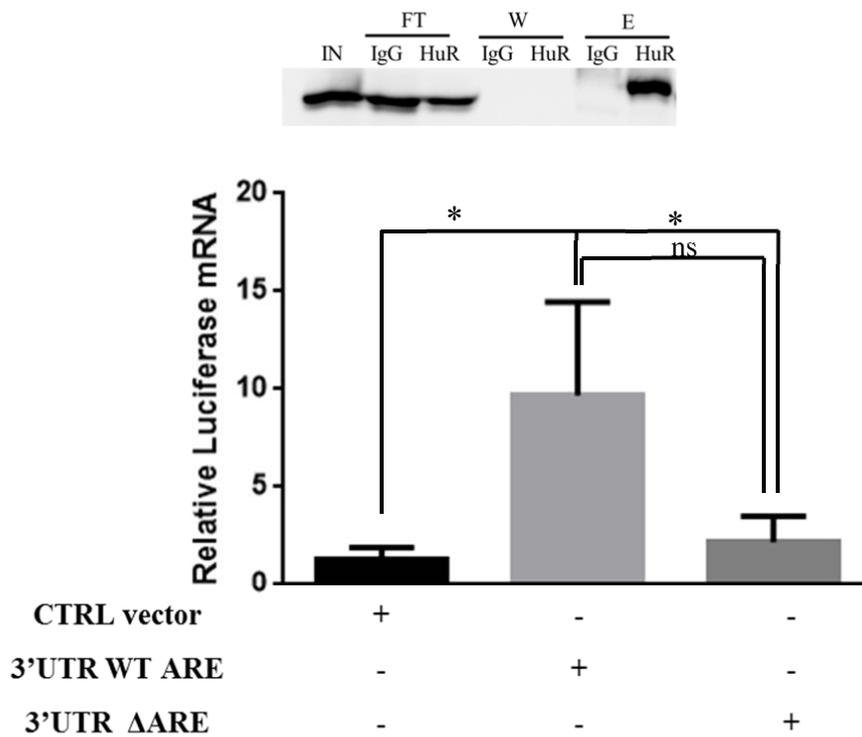


Figure 19. *HuR* interacts with the conserved ARE motif in the AChR γ 3'UTR. A representative western blot quantifying and confirming the precipitation of HuR is shown (top panel: IN, input; FT, flow through; W, wash; E, Elution). (Bottom panel) Luciferase mRNA levels observed after RIP assays performed on COS cells transfected with WT, mutated AChR γ 3' UTR constructs or the empty vector as a control. Samples were immunoprecipitated with either IgG as control, or with an antibody against HuR. RT-QPCR analyses revealed a significantly decreased amount of luciferase mRNA interacting with HuR in the mutated constructs. Data are presented as percentage of control SEM from three to four independent experiments. * $p < 0.05$; ns, not significant; unpaired Mann-Whitney's U test.

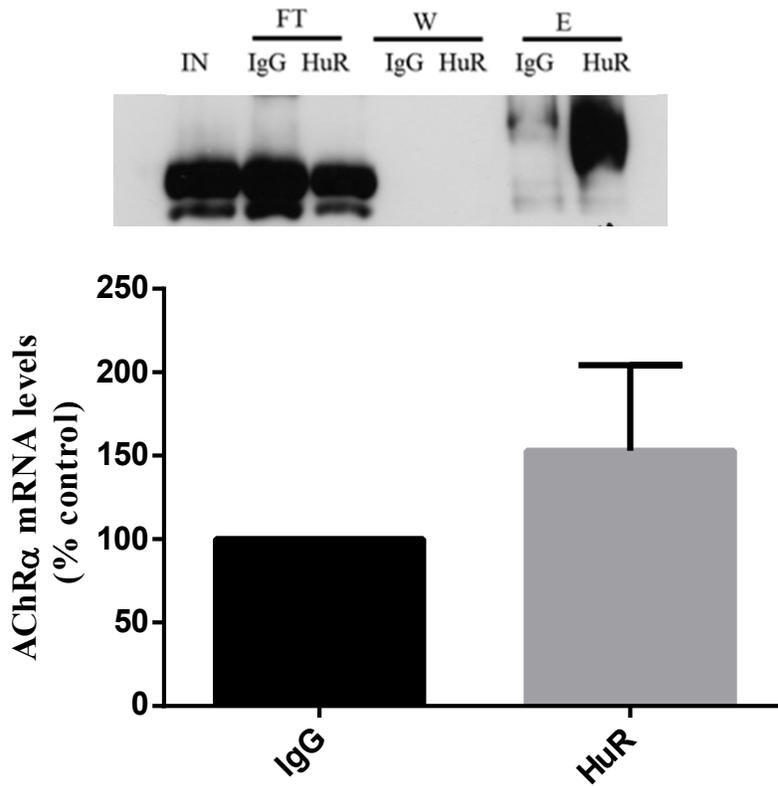


Figure 20. HuR interacts with the AChR α 3'UTR. A representative western blot quantifying and confirming the precipitation of HuR is shown (top panel; IN, input; FT, flow through; W, wash; E, Elution). Luciferase mRNA levels observed after RIP assays performed on COS cells transfected with WT AChR α 3' UTR constructs (bottom panel). Samples were immunoprecipitated with either IgG as control, or with an antibody against HuR. RT-QPCR analyses revealed luciferase mRNA interacting with HuR (n=2).

iii. Testing the functional role of HuR in myotubes by siRNA mediated HuR knock-down.

We have shown that the overexpression of HuR in COS cells, transfected with WT or ARE mutated reporter constructs, increased WT reporter mRNA levels by 200% when compared to pcDNA3 vector control, an effect that was not seen for the mutated construct (Article 1: Figure 4 D and E). If the lack of ColQ is upregulating AChR subunit mRNA through HuR, the absence of HuR should mimic the decrease in AChR mRNA levels observed in the ColQ rescue experiments performed by Sigoillot et al, 2010 (Sigoillot et al., 2010a).

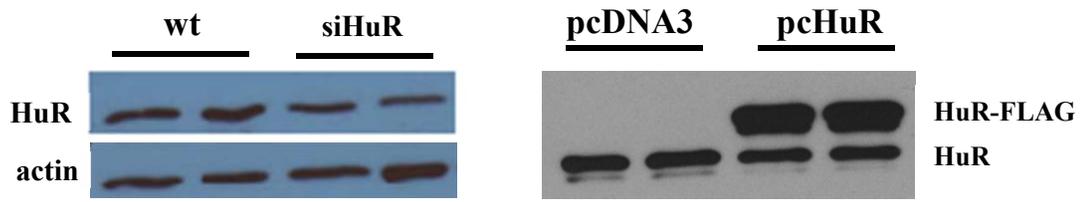
Thus, we sought out to further confirm the functional role of HuR on AChR mRNA in differentiated muscle cells. siHuR was transfected in C2C12 myoblasts 24hrs before differentiation was induced and again 3 days after differentiation. Cells were harvested 5 days after differentiation was induced for RT-QPCR and western blot analysis. The western blot of HuR showed a 20% decrease in HuR protein levels, which was not significant enough to modify the AChR subunit mRNA levels (data not shown). Van der Geissen, et al., have shown an 80% decrease in HuR 48hrs after transfection and by day 3 of differentiation, HuR protein levels were back to normal (van der Geissen et al., 2003). The short half-life of siHuR coupled with the low transfection efficiency of myotubes thus makes it difficult to assess HuR effect on AChR mRNA levels at the optimal time point of differentiation.

Therefore, because of their ease of transfection, T293 (human embryonic kidney cell line) were used to test siHuR. The siHuR, pcDNA3 or pcHuR were co-transfected into the T293, with either the empty luciferase vector or the luciferase construct containing the WT 3'UTR of AChR α or γ (100% transfection efficiency). Cells were harvested for luciferase and western blot analysis after 24hr transfection. The western blot of HuR protein levels show that HuR was successfully over expressed (+200%) and knockdown (-50%) in these cell lines (Figure 21). Overexpression of HuR was found to increase the level of luciferase activity by

~3-fold from the AChR γ construct compared with pcDNA3 vector control but did not appear to alter the AChR α construct. Moreover, the knockdown of HuR protein did not change the luciferase activity for either construct when compared to the control luciferase construct.

Based on the results in figures 20 and 21, preliminary results confirm that 3'UTR of AChR α does not interact with HuR. However, endogenously, HuR interacts with the AChR α transcript in both the MLCL and C2C12 myotubes (Figure 17 and 18). HuR has been found to bind the 5'UTR of multiple transcripts; in this case though HuR acts as a translational repressor (Meng et al., 2005; Yeh et al., 2008). Furthermore, HuR represses alternative splicing of the Fas primary transcript through the binding of its exon (Izquierdo, 2008). Therefore, it is possible that HuR may bind the AChR α subunit transcript outside the 3'UTR and thus may post-transcriptionally regulate the transcript differently from that of the β subunit. Preliminary scans of the AChR α pre-mRNA reveal the presence of the hallmark pentamer AUUUA sequence in the introns but not the 5'UTR. Regardless, the results are still preliminary and further optimization of the experimental set up must be completed to confirm the results.

A.



B.

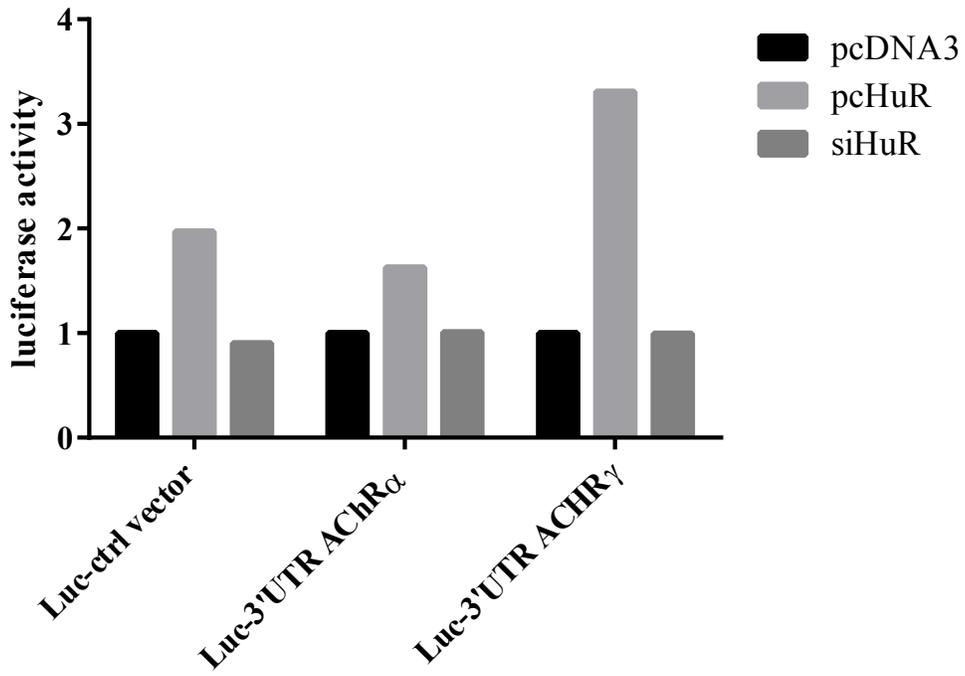


Figure 21. The AChR 3'-UTR and its expression of a reporter construct in human embryonic kidney cells.

A. Representative Western blot quantifying and confirming the knock-down (left) and over expression (right) of HuR in T293 cells.

B. T293 cells were co-transfected with the luciferase reporter construct and either pcDNA3, pcHuR or siHuR. The cells were harvested 24hrs after transfection. Renilla luciferase activity was measured and standardized to the activity obtained with the parental vector and the firefly luciferase activity and expressed in arbitrary units. (n=1).

iv. HuR shuttling between the nucleus and cytoplasm.

Although HuR is primarily localized in the nucleus, it has been reported previously that during myogenesis, HuR shuttles from the nucleus to the cytoplasm, where it performs its function as an mRNA stabilizing protein by binding to such transcripts as those coding for MyoD, myogenin and AChE (Deschenes-Furry et al., 2005; Figueroa et al., 2003). Due to the fact that the absence of ColQ induces an increase in HuR protein and because HuR primarily binds its target mRNA in the cytoplasm, we investigated whether there was an upregulation in its nuclear/cytoplasmic shuttling in the absence of ColQ. To this end, I produced a series of immunofluorescence experiments aimed at comparing the sub-cellular localization of HuR within WT and ColQ-deficient myotubes 5 days after differentiation. As HuR is saturated in the nucleus, we compared the cytoplasmic HuR levels of WT and ColQ deficient myotubes (Figure 22). However, due to the heterogeneity in the length and diameter of myotubes and the dispersion of HuR protein throughout the myotube, it was impossible to assess these levels quantitatively or qualitatively. Thus, to apprehend this mechanism, we will have to switch to cellular fractionation and quantification of cytoplasmic HuR by western blot analysis.

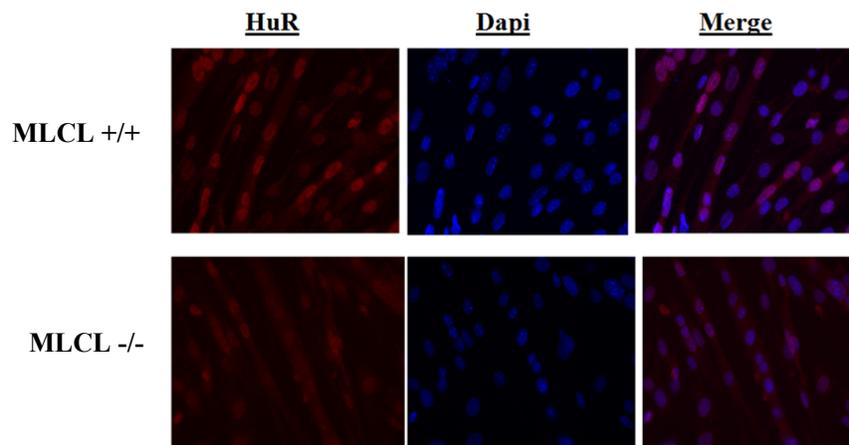


Figure 22. HuR localization in WT (MLCL^{+/+}) and ColQ-deficient muscle cells (MLCL^{-/-}). Myotubes were fixed five days after differentiation and analysed by immunofluorescence staining for DAPI and against HuR.

4- Is p38 the primary pathway inducing the ColQ regulated HuR dependent increase AChR subunit mRNA?

Thus far, we have provided significant evidence that HuR regulates AChR β subunit transcript levels in a manner similar to that of the absence of ColQ. We then explored the intracellular mechanism that links extracellular ColQ to the intracellular activation of HuR. We chose the p38 α MAPK pathway as the plausible mechanism because of its importance in the post-transcriptional regulation observed in ARE containing mRNA, specifically regulating the abundance and/or activity of HuR (Srikantan and Gorospe, 2012). When assessing the p38 pathway with respect to ColQ, we found that the absence of ColQ increases p38 phosphorylation and thus activation. As our antibody recognises α , β , and γ isoforms we could not specify the isoform(s) showing this increase in phosphorylation. Because of the importance of α p38 isoform in HuR regulation, we chose an inhibitor specific to the α/β p38 isoforms (SB203580). We went on to demonstrate that the activation of p38 α/β regulates HuR expression (Article 1: Figure 6). Thus the next step is to determine if the inhibition of p38 α also has an effect on AChR subunit expression.

Post-synaptic gene expression is regulated in part via the ERK1/2 and JNK MAPK pathways (Lacazette et al., 2003; Rimer, 2010). As a result we determined whether these pathways can also affect HuR expression levels.

To determine if the JNK pathway was regulating HuR expression, WT myoblasts were treated with JNK inhibitor (SP600125) 24hrs after differentiation was induced. Myotubes were then treated with the inhibitor every 24hrs until T1 or T2 and HuR protein levels were compared to those in non-treated WT myotubes at the respective differentiation state. The inhibition of the JNK pathway had no effect on HuR protein levels (figure 23). This is not surprising as p38 α activation has been found to inhibit the JNK pathways (Heinrichsdorff et al., 2008).

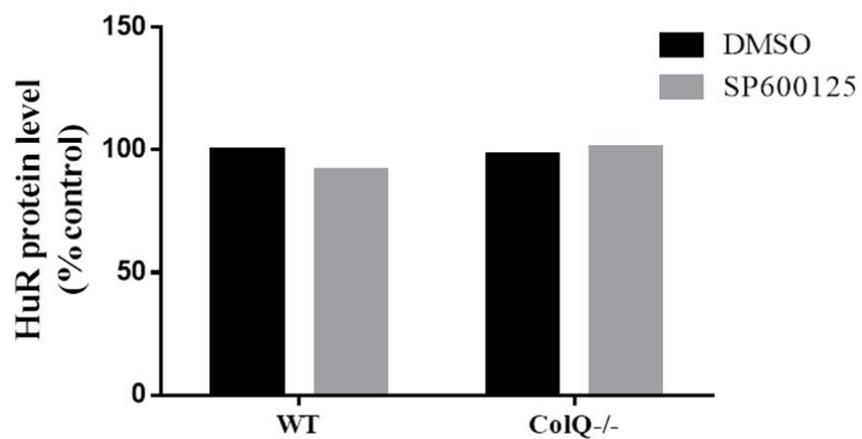
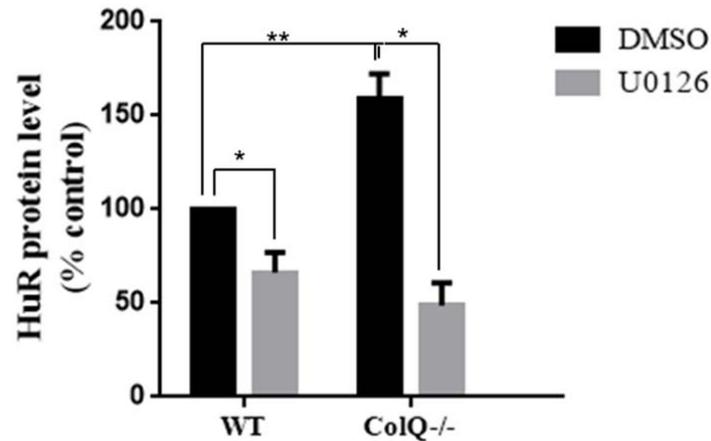


Figure 23. *The inhibition of JNK does not alter HuR in the absence of ColQ.* Western blot of total protein extracts show no difference in HuR protein levels in the presence of JNK inhibitor (SP600125) in WT and ColQ-/- myotubes. HuR protein quantification normalized to GAPDH (n=1).

To investigate if the ERK pathways regulates HuR expression, WT myoblasts were treated with ERK1/2 inhibitor U01206 24hrs after differentiation was induced. Myotubes were then treated with the inhibitor every 24hrs for 5 days after differentiation was induced and HuR protein levels were compared to those in non-treated WT myotubes at the respective differentiation state (Figure 24A). Western blot results showed that the inhibition of ERK1/2 activity resulted in a 41.5% and 44.5% decrease in HuR protein levels in WT and ColQ^{-/-} myotubes respectively. The next step would be to determine if this loss of HuR has an effect on AChR mRNA levels. Using RT-QPCR we have found a decrease in the AChR β subunit (figure 24B). But because ERK1/2 activation is generally required for AChR subunit expression, we cannot determine if the decrease in AChR subunit mRNA levels is due to the loss of ERK1/2 dependent expression or loss of HuR stability. To test this, we could transfect plasmids containing the AChR subunit genes without their respective promoters to determine if the inhibition of ERK1/2 still has an effect on mRNA levels.

Therefore, we can conclude that the JNK pathway is not involved in the ColQ regulated activation of HuR, however the role of ERK1/2 is still unclear.

A



B

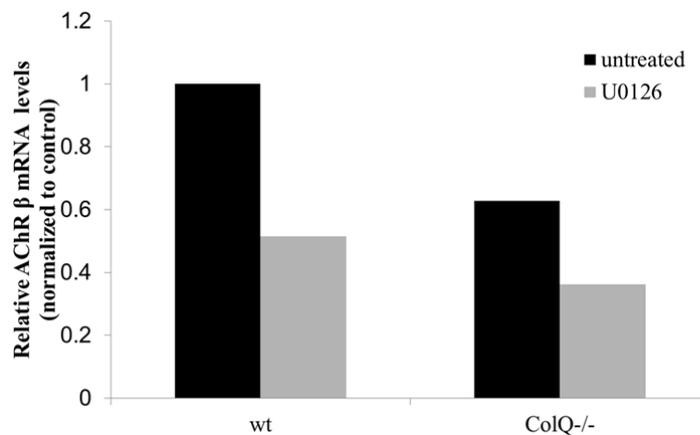


Figure 24. The inhibition of ERK1/2 prevents the upregulation of HuR in the absence of ColQ.

A. Western blot of total protein extracts show a significant decrease in HuR protein levels in the presence of ERK1/2 inhibitor (U0216) in WT and ColQ^{-/-} myotubes. HuR protein quantification normalized to GAPDH. Data are presented as percentage of control SEM from three independent experiments. (n=3) *p<0.05; **p<0.01 unpaired Mann–Whitney’s U test. B. Quantification by RT-QPCR shows a decrease in AChR β mRNA levels in the presence of ERK1/2 inhibitor (U0216) in WT and ColQ^{-/-} myotubes. AChR β mRNA quantification was normalized to GAPDH.

5- Is the ColQ dependent upregulation of AChR mRNA *in vivo* also regulated by HuR?

i. ColQ's effect on AChR Subunit mRNA is chronic.

As previously mentioned, our lab was the first to show that ColQ regulates postsynaptic differentiation (Sigoillot et al., 2010a). Specifically, this article exemplifies that ColQ deficiency leads to an *in vivo* upregulation of mRNAs coding for α , β , and ϵ AChR relative to GAPDH mRNAs in gastrocnemius muscle from P7 ColQ^{-/-} mice. Yet, this upregulation was only significant for the β AChR subunit (+63% and +106% respectively).

To understand how the ColQ mediated AChR subunit mRNA levels are affected over time, we have started expanding the analysis into the adult mouse at P30. A significant increase in mRNA levels of α and β AChR subunits (+56% and +27% respectively; Figure 25) was observed in the ColQ^{-/-} mice at this age. Therefore, it appears as though ColQ's effect on AChR mRNA levels is long term and appears to be more pronounced with age.

Due to the upregulation of AChR mRNA seen at P7 and P30, it would be interesting to determine if endogenous HuR is interacting with AChR mRNA *in vivo*. Once this is confirmed, it would be interesting to see if knock-down studies of HuR using siRNA against HuR affect AChR subunit mRNA levels.

We had initially tried using siRNA targeted against HuR *in vitro* but had run into some difficulties due to the time frame of the experiment. *In vivo* should be more adequate and would involve the injection of naked siHuR into the great saphenous vein of mice and further quantification of AChR subunit mRNA 48hrs after. Venous injection delivers siRNA to all limb muscles with minor cell damage and results in a 95% decrease in gene expression two days post-injection (Hagstrom et al., 2004).

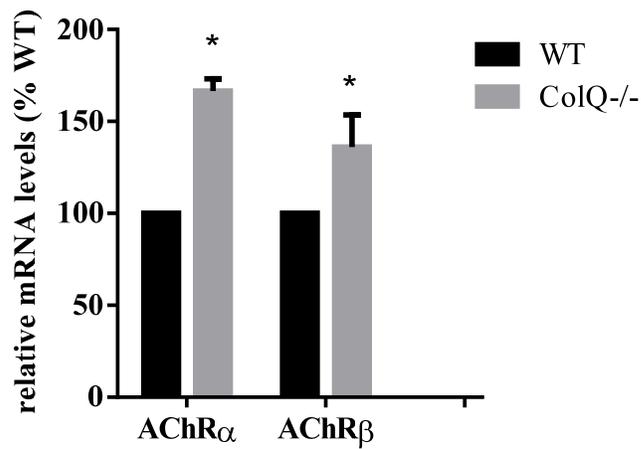


Figure 25. ColQ regulates AChR mRNA levels at adult NMJs. Quantification of AChR subunits mRNAs on wt and ColQ^{-/-}P30 gastrocnemius muscles by real-time RT-PCR. Levels of mRNAs are represented as relative expression. AChR subunit mRNA were normalized to GAPDH (n=3 independent experiments).

ii. HuR protein levels remain unchanged *in vivo* in the absence of ColQ.

Previously, it was demonstrated that cultured ColQ deficient myoblasts that differentiated over five days, exhibited a 50% increase in total HuR protein levels when compared to WT. Therefore, we wanted to see if the absence of ColQ also induced an upregulation of HuR *in vivo*. Interestingly, HuR protein levels remained constant in the ColQ^{-/-} mice at P30 when compared to WT mice of the same age (figure 26).

HuR is ubiquitously expressed and stored predominantly in the nucleus. When a cell is stimulated in response to apoptosis, cell division, and myogenesis, in most cases HuR expression is not upregulated. Rather the HuR stored in the nucleus is rapidly translocated to the cytoplasm, where it interacts with and regulates the stability of its target mRNA (von Roretz et al., 2011). Therefore, it could be deemed that *in vivo*, ColQ is not regulating the expression of HuR, but rather the shuttling of HuR from the nucleus to the cytoplasm.

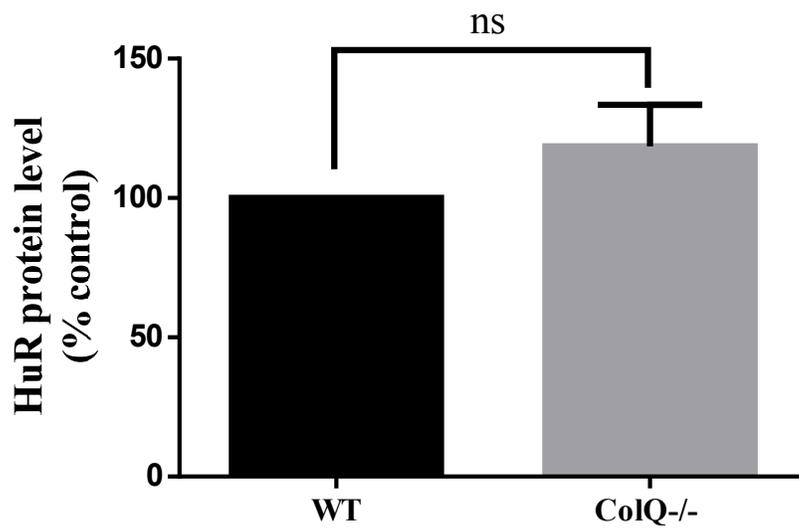


Figure 26. *The absence of ColQ does not alter HuR protein levels in vivo. Western blot of total protein extracts show no significant increase in HuR protein levels in the absence of ColQ in vivo at P30. HuR protein quantification normalized to GAPDH. Data are presented as percentage of control SEM from four independent experiments. (n=4). ns, not significant.*

ARTICLE 1

ColQ regulates Acetylcholine receptor mRNA stability through HuR and the activation of p38: implications for Congenital Myasthenic Syndrome with Acetylcholinesterase deficiency

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ABSTRACT

COLQ mutations in the human gene, are causative for a congenital myasthenic syndrome with AChE deficiency. ColQ is a specific collagen that anchors acetylcholinesterase (AChE) in the synaptic cleft of the neuromuscular junction (NMJ). Besides its interaction with AChE, ColQ also binds to MuSK, a muscle tyrosine kinase essential for NMJ formation. In *COLQ* knock-out mice, a valid model for this CMS pathology, as well as in a muscle cell line deficient for ColQ, the lack of ColQ induces an increase in the levels of Acetylcholine receptor (AChR) subunit mRNAs, a process that is mediated by MuSK. This mechanism probably represents an adaptation to high levels of Acetylcholine. Here, we decipher the mechanisms that drive AChR mRNA upregulation in the absence of ColQ and the pathways linking ColQ to the AChR RNA metabolism. We show that the levels of AChR mRNAs are post-transcriptionally regulated by an increase in their stability. We demonstrate that the lack of ColQ induces an increase in HuR, an RNA binding protein, and that HuR binds the AU-rich element within the AChR β -subunit transcript. This HuR/AChR transcript interaction is responsible for the AChR β -subunit mRNA stabilization. More, ColQ's effect on HuR levels is at least partially mediated by MuSK and p38 activation. Thus, we provide insight into the muscle signaling pathways that are affected by ColQ deficiency leading to the myasthenic syndrome with AChE deficiency. These results open the door to a new therapeutical approach of this disease.

INTRODUCTION

The Collagen ColQ, plays a critical role at the mammalian neuromuscular junction (NMJ) through the anchoring of Acetylcholinesterase (AChE) in the synaptic basal lamina (1, 2). ColQ is expressed from a single gene and contains several domains including an N-terminal peptide, a central collagen domain, and a C-terminal peptide. The collagen domain of ColQ monomers associate to form a triple helix with the N-terminus of each branch interacting with the C-terminus of the AChE splice variant AChE_T (2, 3). The central collagen domain contains two clusters of basic residues that supposedly bind the proteoglycan perlecan (4-6), which in turn binds to the transmembrane protein dystroglycan (7). Furthermore, the C-terminal region of ColQ binds MuSK, and localizes the ColQ/AChE complex at the synapse (8). To date, over 30 mutations have been identified in the human COLQ gene, all of which leading to a congenital myasthenic syndrome with endplate AChE deficiency (9-11). CMS with AChE deficiency is first detected in childhood and is characterized by muscle weakness, muscle atrophy, and slow pupillary response to light stimulation. As the disease progresses, skeletal deformities (e.g., lordosis or scoliosis), ptosis, ophthalmoplegia, and difficulty breathing can occur with a high risk of lethality (12). A mouse model of CMS with AChE deficiency has been generated by the knock-out of the COLQ gene portraying most of the characteristic features observed in patients suffering from CMS with AChE deficiency (13). Furthermore, restoring ColQ in these mutant mice has been shown to restore a normal phenotype in the motor functions, synaptic transmission and structure of the NMJ (14). Thus, the ColQ mutant mouse is a valid model to decipher the role of ColQ in NMJ functioning as well as to understand the pathophysiology of CMS with AChE deficiency. Recently, we have demonstrated that besides its structural function, ColQ is also a signalling protein that regulates AChR cluster density and synaptic gene expression via its interaction with a transmembrane tyrosine kinase called MuSK (15). The clustering of AChR in the membrane of the myofiber is first induced by MuSK localized in the central region of the muscle (16-18). Then, upon muscle innervation, the neuron releases a neural isoform of the glycoprotein Agrin, which binds LRP4, the co-receptor of MuSK (19, 20). Agrin binding strengthens the LRP4/MuSK interaction and its signal is passed through the MuSK/ Dok7 complex resulting in the Dok7 dependent phosphorylation of MuSK. Then, the phosphorylation of MuSK recruits the cytoplasmic protein rapsyn and induces the clustering of AChR in the post-synaptic domain (21). Specifically, we have shown that ColQ deficiency increases the density of AChR as a result of AChR mRNA upregulation. The absence of ColQ increases all five AChR subunit mRNAs, a process that probably represents an adaptation to higher levels of Acetylcholine (15). One of the mechanisms regulating mRNA levels is through the regulation of their stability by trans-acting factors such as RNA binding proteins (RBPs) or microRNAs. Previous studies have reported that Human antigen R (HuR) a member of the ELAV/Hu family of RBPs is critical during skeletal myogenesis and post-synaptic NMJ formation. HuR has been known to coordinate the regulation of muscle early and late onset differentiation genes including myogenin, MyoD and AChE (22, 23). Therefore, we explored the possibility that ColQ could control AChR mRNA stability through a mechanism mediated by HuR.

In this study, we provide insight into the mechanism that regulates ColQ mediated AChR subunit expression. Since the AChR β subunit is essential for AChR clustering, we focused on the metabolism of β subunit mRNA. We found that (1) the absence of ColQ increases AChR β subunit mRNA stability and upregulates HuR (2) using RNA immunoprecipitation assay, HuR interacts specifically with its ARE (AU-rich elements, AREs) to positively regulate AChR β subunit expression (3) ColQ controls HuR levels at least partially by p38 activation and MuSK.

RESULTS

Agrin has been shown to induce the phosphorylation of the AChR β subunit via rapsyn (24). Mutations of the β subunit phosphorylation site impairs agrin-induced cytoskeletal anchoring and AChR aggregation *in vitro* (25) and mice deficient for AChR β subunit tyrosine phosphorylation have abnormal neuromuscular junctions with respect to their structure, size and AChR density (26). Thus because AChR β plays an important role in the postsynaptic clustering of the receptor and the overall shape of the synapse, we focused our study on this subunit.

The lack of ColQ induces an increase in AChR subunit mRNA stability

Among the possible mechanisms of ColQ mediated regulation of AChR β subunit mRNA, we tested the influence of ColQ on transcript stability. To investigate this mechanism, we used the WT and the ColQ-deficient muscle cell lines generated in our laboratory from P7 WT and ColQ knockout mice respectively (8). WT and ColQ-deficient myoblasts were plated in growth medium and 24 h later (at confluence) transferred to differentiation medium (DM). Two days after myotube formation, cultures were then treated with 4 μ g/ml of the transcriptional inhibitor actinomycin D and total RNA was extracted at different time points (0, 4, 6 and 8 h). mRNA was isolated, reverse-transcribed (RT) and further quantified by QPCR. Quantification of the specific mRNA at the different time points showed that AChR β subunit mRNA levels in WT and ColQ-deficient cells were significantly different after 4h and 6h of treatment. For these 2 time points, degradation was significantly lower in the absence of ColQ when compared to WT (Figure 1). This indicates that the absence of ColQ increased AChR mRNA stability which correlates with the previously observed increase in AChR mRNA levels.

HuR is a candidate protein for ColQ dependent AChR transcript stability

The next question raised by the findings described above concerned the identity of trans-acting factors that can influence the ColQ dependent stability of AChR subunit mRNA. In this context, it has been shown that the ubiquitously expressed HuR protein plays a critical role in differentiating muscle cells by interacting with the AU rich element (ARE) contained in the 3'UTR of target mRNA leading to their stabilization (23). To explore a possible link between ColQ and HuR, we compared the levels of HuR mRNA in WT and ColQ-deficient muscle cells. Microarray studies were completed during various stages of WT and ColQ-deficient myotube differentiation, with time points defined as T1 T2 and T3. The T1 time point is defined by the presence of newly formed myotubes. The T2 time point is observed 2 days after T1 and characterized by the appearance of AChR clusters. Three days later, T3

is marked by clusters of AChE-ColQ and the first spontaneous muscle contractions. In agreement with the previously described role of ColQ, the ColQ-deficient cell line does not present any AChE clusters (13). In the absence of ColQ, an increase in HuR mRNA was observed at all time points (T1, +1.85; T2, +1.70; T3, +1.63), with a significant increase detected during early myotube formation at T1 (Figure 2A). HuR protein quantification showed a similar result in the absence of ColQ resulting in a significant increase in HuR in two of the three stages of myotube differentiation with a 40% increase at T1 and a 60% increase at T2 (Figure 2B and 2C).

When performing a bioinformatics analysis using the online data base AREsite to identify classic ARE sites (27), we found that AChR β and two other subunit mRNA contain at least one computationally predicted HuR site in their 3'UTR (see supplementary Material, Figure. S1A). The 3' UTR of the mouse AChR β transcript is 531 nucleotides long with one ARE site identified. Sequence comparison among human, orangutan and mouse reveals that this site is conserved across mammals (supplemental Material Figure S1B). Based on this, we hypothesized that the stabilization of AChR β subunit RNA could be dependent on HuR and mediated by its binding on the conserved ARE site present in the 3'UTR AChR transcript.

HuR interacts with ARE element located in the 3'UTR of the AChR beta transcript

In order to test an endogenous interaction between HuR and AChR β transcripts, we performed RNA immunoprecipitation (RNA IP) experiments with cultured myotubes. After formaldehyde cross-linking and HuR or IgG (negative control) immunoprecipitation, co-immunoprecipitated mRNAs were analyzed by quantitative RT-QPCR (Figure 3). Our results clearly show that in myogenic cells, HuR indeed interacted with AChR β subunit mRNA containing an ARE site and not with the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) that has no ARE sites.

In order to evaluate the contribution of the 3'UTR ARE in the AChR/ HuR interaction, we cloned both a WT and ARE mutated form of the full-length 3' UTR of the mouse AChR β subunit transcript and inserted it downstream of the luciferase reporter gene (3'UTR WT ARE and 3'UTR Δ ARE respectively) driven by a constitutive promoter (Figure 4A). The mutated clone contains three point mutations in the ARE element which should prevent it from recruiting HuR.

We then individually transfected these constructs into COS cells which already express endogenous HuR (28). RNA IP analysis, completed 48hr after transfection showed a significant interaction of the 3'UTR WT construct when compared to that of the empty vector (Figure 4B, top and bottom panel). Mutation of the conserved ARE (3'UTR Δ ARE) resulted in a significant 13 fold decrease in the binding of HuR to the AChR β full length 3'UTR, with measured abundance of reporter mRNA in the immunoprecipitates being similar to that of the empty vector. Taken together these results showed that HuR interacts with the conserved ARE element in the 3'UTR of AChR β subunit (Figure 4B).

HuR mimics the mRNA regulatory effect seen by ColQ

If ColQ is regulating AChR subunit mRNA through HuR, the presence of HuR should mimic the increased AChR mRNA stability observed in the absence of ColQ. Thus to directly assay for the role

of HuR after its recruitment to the ARE element in the 3'UTR of AChR β , COS cells were transfected with 3'UTR WT ARE or 3'UTR Δ ARE constructs. Cells were harvested 48hrs and the Luciferase reporter transcript levels were measured by RT-QPCR (Figure 4C). The mutation in the ARE motif led to a 37% decrease in reporter transcript levels compared to the WT construct suggesting that the endogenous level of HuR present in COS cells was not able to stabilize the mutated transcripts. Furthermore, over-expression of HuR in COS cells transfected with the 3'UTR WT ARE construct was found to increase WT reporter mRNA levels by 200% when compared to pcDNA3 vector control. The mutant reporter construct on the other hand presented no alteration in its mRNA levels in the presence of HuR overexpression (Figure 4D,E).

To complement these experiments, we also compared the mRNA stability of the two constructs transfected into COS cells. To this end, 48hrs post transfection, we subsequently inhibited transcription by adding actinomycin D, and then measured the relative abundance of luciferase mRNA at 0, 2, 4 and 6h thereafter (Figure 4F). As shown in Figure 4F, the degradation rate of the mutant ARE luciferase construct mRNA was significantly increased when compared to that of the control. Taken together these results indicate that HuR binds the ARE element in the 3'UTR of AChR β , stabilises the transcript and as a result increases the AChR β mRNA levels.

ColQ regulates HuR expression through the activation of p38

Thus far, we have provided significant evidence that HuR regulates AChR β subunit expression in a way that is similar to that of the absence of ColQ. We now wanted to understand the link between HuR to ColQ itself by determining the intracellular mechanism that connects the extracellular collagen ColQ to the intracellular RNA-binding protein HuR. We have previously shown that ColQ increases synaptic gene expression through its interaction with MuSK (15). Thus we tested if the absence of MuSK also affects the levels of HuR. Using a MuSK deficient muscle cell line, we were able to show by western blot analysis that the absence of MuSK resulted in a 37% decrease in HuR protein levels (Figure 5A and B).

The p38 MAPK pathway is known to play an important role in post-transcriptional regulation observed in ARE containing mRNA, specifically regulating the abundance and/or activity of HuR (29). Furthermore, three isoforms of p38 (α , β and γ) are known to be indispensable for muscle cell differentiation (30). Thus, to determine whether the absence of ColQ induces the activation of p38 signaling at the NMJ we first compared the phosphorylation state of p38 in WT and ColQ-deficient myotubes. Three days after differentiation was induced, western blotting demonstrated that ColQ-deficient myotubes had a 274.4% increase in phosphorylated p38 when compared to that of the WT (Figure 6A and B). To directly assay for the role of the p38 pathway in the regulation of HuR expression, WT myoblasts were treated with the p38 α/β inhibitor SB203580 24hrs after differentiation was induced. Myotubes were treated with SB203580 every 24hrs until T1 or T2 and HuR protein levels were compared to those in non-treated WT myotubes at the respective differentiation state (Figure 6C and D). Western blot results showed that the inhibition of p38 activity resulted in a 15%

and 48% decrease in HuR protein levels in myotubes at T1 and T2 respectively. Thus, we conclude that the absence of ColQ induces the phosphorylation of p38, whose activity is involved in HuR upregulation.

DISCUSSION

A class of CMS result from mutations in proteins localized in the synaptic extracellular matrix (sECM) (31). So far, three causative genes, laminin, agrin and ColQ have been identified in this pathological context. ColQ is a specific collagen that anchors AChE in the ECM (1, 2). The expression of this complex is mostly restricted to the synapse by ColQ interaction with MuSK, a tyrosine kinase receptor that is specifically localized in the postsynaptic domain (8, 32). The absence of ColQ or its low expression induces an increase in ACh in the synaptic cleft as a consequence of AChE deficit. It also leads to muscle intracellular modifications of synaptic protein levels revealing that ColQ directly or indirectly stimulates the activity of signaling pathways. As shown previously by our team, the most striking effect of the absence of ColQ is an upregulation of all AChR subunit mRNAs correlated by an increase in their protein levels (15), a process that may be interpreted as an adaptation to the overload of ACh in the synaptic cleft. Here, we show that a post-transcriptional mechanism of AChR mRNA stabilization is responsible for the increased levels of AChR β mRNAs. In addition, the absence of ColQ increased the mRNA and protein levels of HuR, a RNA binding protein known to stimulate mRNA stability. Furthermore, we show that HuR is capable of binding to conserved ARE elements in the 3'UTR of AChR β subunit mRNA. HuR's interaction with AChR β mRNA increased the stability of this transcript, resulting in an increase in mRNA levels. We provide evidences that ColQ regulates HuR mediated AChR β subunit stability through MuSK and that the p38 signalling pathway control the levels of HuR in a ColQ dependent manner.

AChR mRNA levels are regulated by post-transcriptional mechanisms in the absence of ColQ

To date, the transcriptional regulation of AChR mRNA has been described as the major mechanism for AChR regulation. AChR transcription is regulated through three pathways triggered by the neurotrophic factors, agrin and neuregulin. These synaptic molecules induce the MAPK kinase (ERK), the PI3K and the JNK signaling pathways (33, 34), which converge on the activation of the Ets transcription factor, GABP. Another member of the Ets family Erm has also been involved in the regulation of subsynaptic gene expression *in vivo* (35) but only GABP was been shown to bind an N-box in the AChR subunit promoters inducing their transcription in synaptic nuclei (36, 37).

Our study provides the first evidence that in addition to transcriptional regulation, post-transcriptional events are also capable of modulating the expression of AChR subunits. In the present study, we found that AChR β mRNAs are a direct post-transcriptional target for the stabilizing effects of HuR. Although only, two (γ, δ) of the 4 AChR subunits contain classic AREs, we have evidence showing HuR binding to AChR subunit mRNAs which lack these classic elements. Interestingly, a strong interaction between HuR and the AChR γ mRNA as well as AChR α mRNA is present (data not

shown) with the latter mRNA not containing a canonical ARE in its 3'UTR. We further demonstrated HuR interaction with the ARE in the 3'UTR of the AChR γ subunit using reporter construct containing the WT or mutated ARE in the γ subunit 3'UTR (Suppl data S2). The reporter constructs containing the 3'UTR of the α subunit mRNA confirmed that HuR binds to endogenous α subunit mRNA but the location of HuR's binding site within this transcript remains to be identified. Besides the known AU-rich elements described as the traditional HuR binding site motif, other U rich or undetermined elements have been shown to bind this protein (38, 39). Overall, it is thus very likely that the ColQ dependent regulation of the remaining 4 AChR subunits (α , δ , ϵ and γ) is also a post-transcriptional stabilisation process by HuR.

We previously showed that synaptic mRNA levels, specifically those of the AChR subunit mRNA are differentially affected by ColQ deficiency (15). The amplitude of the effect depends on the specific subunit, the differentiation stage of muscle cells *in vitro* and the developmental stage *in vivo*. One explanation concerning the ColQ dependent effect on a specific AChR subunit mRNA could be related to the number of ARE in a specific 3'UTR. However, although the δ 3'UTR has two ARE compared to one in the β 3'UTR, the upregulation level is comparable for the two mRNAs in ColQ-deficient cells *in vitro* (15). Another explanation would be that in addition to HuR, other trans-acting factors could target specific AChR subunit mRNAs. HuR is capable of acting together or in competition with other RBPs and microRNAs depending on the environmental context. This can be achieved through binding to ARE nearby sequences in the 3'UTR or to the ARE site itself through competitive or co-binding mechanisms (40). Furthermore, we cannot exclude that transcriptional events contribute to the AChR mRNA levels in absence of ColQ. Yet the transcriptional regulation of these subunits is also differentially regulated with the upstream flanking regions of the AChR subunit genes containing such DNA elements as TATA and CAAT boxes, Sp1 binding sites and SV40 core enhancer sites (41). Thus AChR specific mRNA levels could be the result of a complex regulation of transcriptional and post-transcriptional mechanisms.

The mRNAs coding for the synaptic proteins MuSK and rapsyn are also upregulated in the absence of ColQ (15). When screening the 3'UTR sequences of MuSK and rapsyn, only MuSK mRNA contained an ARE site. Although we cannot exclude the existence of non-canonical HuR binding sequence in rapsyn mRNA, this could suggest that a restricted number of these synaptic mRNAs are controlled by HuR and that AChR mRNAs coding the 5 subunits and potentially other mRNAs such as MuSK mRNAs are coordinately regulated by the same mechanism. It should also be noted that MyoD and Myogenin which have been shown to bind HuR (23) are not upregulated in the absence of ColQ (S. Sigoillot, F. Bourgeois, J. Karmouch, E. Krejci, C. Chevalier, R. Houlgatte, J. Leger, and C. Legay, in preparation). AChE mRNAs have also been identified as targets of HuR (22). However, their increase is only significant at one time point of myotube differentiation *in vitro* (S. Sigoillot, F. Bourgeois, J. Karmouch, E. Krejci, C. Chevalier, R. Houlgatte, J. Leger, and C. Legay, in preparation).

HuR mediates AChR mRNA stability and p38 regulates HuR levels.

Here, we demonstrated that HuR is upregulated in the absence of ColQ and that p38 signalling pathway is involved in this regulation. HuR expression is known to be controlled by the transcription

factor NF-kappaB that binds directly to the HuR promoter and by the TGF/Smad pathway (42, 43). Interestingly, consensus binding sites for NF-kappaB have also been found in the rapsyn promoter (44). More, overexpression or downregulation of this transcription factor is paralleled by an increase or a decrease respectively in rapsyn mRNA and protein (44). It is therefore possible that NF-kappaB regulates Rapsyn expression as well as other genes such as HuR.

In search for signalling pathway induced by the absence of ColQ, we reveal that the absence of ColQ increases the phosphorylation and thus the activation of p38 which correlates with HuR upregulation. The mechanisms by which p38 activation control HuR levels remain to be elucidated. Direct phosphorylation of HuR by p38 α induced HuR translocation to the cytoplasm where it interacts with its target mRNA (45) but there is no evidence that p38 could also regulate HuR levels. On the other hand, p38 α isoform has also been found to activate NF- κ B (46) which could in turn stimulate HuR expression.

We have previously demonstrated that ColQ dependent regulation of AChR subunit mRNA relies on its interaction with MuSK (15) and here, we show that MuSK deficiency affects HuR levels. Along this line and according to our data, ColQ's inhibitory effect on HuR expression could be at least partially mediated by MuSK. It also suggests that the lack of ColQ favors the interaction of MuSK with partners that stimulate HuR expression. So far, none of the protein binding MuSK intracellular domain have been implicated in a HuR transcriptional pathway. It should also be noted that the upregulation of HuR and AChR mRNAs occurs *in vitro* in the absence of agrin, an activator of MuSK indicating that agrin cannot be part of the MuSK dependent control of HuR. Wnts also bind MuSK and are expressed by muscle cells *in vitro* and *in vivo*. In this context, we cannot exclude the possibility that the lack of ColQ interferes with the Wnts binding affinity on MuSK and downstream events. MuSK activation has been shown to activate several signalling pathways including the PI3K, MAPK (ERK) and JNK pathways but not the p38 pathway. However, again, these pathways were only described in response to agrin and neuregulins which are absent in our experimental conditions. Thus, considering the link between ColQ and the p38 pathway, two alternatives are possible, either ColQ regulates a p38/MuSK dependent pathway or the activation of p38 is activated by a ColQ dependent indirect mechanism.

One of the hallmark of ColQ-deficient NMJ is the perturbation of the extracellular matrix organization that is illustrated on electron micrographs with Schwann cells processes intruding into the synaptic cleft (13). This modification may induce a cellular stress and p38 has been reported as a major stress response induced signaling pathway. The stress response could be mediated by postsynaptic transmembrane receptors such as integrins, which are major sensor of mechanical forces in muscle. The α 7 β 1 integrin is enriched at the post-synaptic membrane of the neuromuscular junction and is upregulated in patients with Duchenne muscular dystrophy and *mdx* mice where the attachment of the skeletal muscle to the extracellular matrix is altered (47, 48). However, α 7 β 1 integrin levels are not significantly affected either *in vitro* or *in vivo* (S. Sigoillot, F. Bourgeois, J. Karmouch, E. Krejci, C. Chevalier, R. Houlgatte, J. Leger, and C. Legay, in preparation) suggesting that integrins might not be involved in the p38 activation in response to ColQ deficiency.

In this context it will be interesting to explore the activity of the p38-HuR pathway in other myasthenic syndromes, with particular focus on the synaptic class of these diseases including laminin induced

myasthenic syndromes. Indeed, the same morphological defects have been observed in laminin mutants (49). Here, it should point out that several components of the ECM such as perlecan and biglycan are indirectly and directly binding MuSK (6, 50) and thus MuSK could also act as a “sensor” of ECM integrity.

HuR, p38 and synaptic gene expression: opening a new approach to neuromuscular diseases.

A similar scenario where HuR and p38 pathway are recruited in a neuromuscular disease is found in spinal muscle atrophy (SMA). This disease is characterized by the progressive loss of motor neurons and consequently muscle atrophy. One of the hallmarks in this disease is the disruption of the NMJ (51, 52). The causative gene for the disease, survival motor neuron (SMN) has been identified (53). SMN mRNAs are stabilized by HuR and activating p38 leads to increased levels of cytoplasmic HuR and thus an increased level SMN protein (54). In Human, there are two similar genes coding for *SMN*, *SMN1* and *SMN2*. Most of the patients lack *SMN1* gene whereas *SMN2* gene is expressed at low levels. Recently, a therapy using celecoxib, an activator of p38, has been proved to increase the levels of SMN proteins by activating the p38 pathway (55). In our study, the levels of HuR are upregulated by p38 activation and therefore similar therapeutic approaches could be explored in ColQ-deficient mice and possibly in other myasthenic syndromes.

MATERIALS AND METHODS

RNA extraction and RT-PCR

Total RNA was extracted from COS-7 and muscle cells using TRIzol reagent (Invitrogen) as recommended by the manufacturer. TRIzol extracted RNAs were treated for 30min at 37°C with DNase followed by 10min at 65°C with DNase STOP (Promega kit) to eliminate possible DNA contamination. cDNAs were synthesized from DNase-treated RNAs using the reverse transcriptase (MuLV; Life technology). Real-time quantitative PCR was performed in triplicates on a 384-well plate with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Using a QuantiTect SYBR Green PCR kit (QIAGEN, Valencia, CA, USA) according to the manufactures instructions, amplifications were performed with the following primer sequences: AChR beta forward, 5'-CCTGAGCCGCTATCCGGAC-3' reverse, 5'-TTGGCCTTCGGCTTCCGACC-3'; Luciferase forward, 5'-AAACCATGACCGAGAAGGAG-3' reverse, 5'-GTCCACGAACACAACACCAC-3' GAPDH QuantiTect primer was purchased (Qiagen).

Applied Biosystems Microarrays

Microarray analysis was run as three independent triplicates per time point (T1, T2 and T3) and per cell line (wt and ColQ^{-/-} cells) using Applied Biosystems Mouse Genome Survey Microarrays, containing probes representing approximately 32,000 mouse genes from the public and Celera databases. Digoxigenin-UTP labeled cRNA was generated from 500 ng of total RNA using Applied Biosystems Chemiluminescent RT-IVT labeling kit and was purified according to the manufacturer's instructions. Microarrays were prehybridized at 55°C for 1 h in hybridization buffer with blocking

reagent. Labeled cRNAs were then hybridized to each array at 55°C for 16 h. Following hybridization, the arrays were washed with hybridization wash buffer and chemiluminescence rinse buffer. Chemiluminescence detection, image acquisition and analysis were performed using Applied Biosystems Chemiluminescence Detection Kit and Applied Biosystems 1700 Chemiluminescent Microarray Analyzer following the manufacturer's protocol. Single-color chemiluminescent signals were quantified, corrected for background and spatially normalized. Applied Biosystems Expression System software was used to extract assay signal, and assay signal to noise ratio values from the micro-array images. Raw data was normalized using Lowess.

RNA immunoprecipitation (RIP)

Muscle cells or COS-7 cells were cross-linked with 1% formaldehyde in PBS for 10 min at room temperature. The reaction was stopped with a wash in ice-cold PBS and resuspended in RIPA buffer. Cross-linked complexes were solubilized in a sonicator bath by six 30-s sonication pulses, and cellular debris were removed by centrifugation (14,000 *g* for 10 min at 4°C). Equal amounts of whole cell extracts were precleared with a Protein A/G plus beads (Santa Cruz) previously blocked with 200 µg/ml of competitor tRNA, and 40 µg/ml of salmon sperm DNA. Complexes were immunoprecipitated using 3 µg of of HuR or IgG antibodies and protein A/G plus beads overnight at 4°C. After three washes with RIPA buffer and two washes with TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA), beads containing the immunoprecipitated samples were resuspended in 100 µl of elution buffer (50 mM Tris-HCl, pH 7.0, 5 mM EDTA, 10 mM DTT, and 1% SDS). Cross-linking was reversed at 70°C for 5 h. Samples were taken throughout the protocol for RNA extraction and further RT-QPCR and western blotting to confirm the efficiency of immunoprecipitation.

Muscle cell lines, culture and transfection.

The generation of the wild type (WT), ColQ-deficient (ColQ^{-/-}) MuSK-deficient (MK^{-/-}) muscle cell lines has been previously described by Cartaud et al. (2004) and Herbst and Burden (2000). Myoblasts were maintained as myoblasts in growth medium supplemented with 10% fetal bovine serum, 20% horse serum, 2 mM glutamine, 2% penicillin/streptomycin (5000 U) and 20 U/ml γ -interferon (Roche Diagnostics) at 33°C in 8% CO₂. Cells were induced to differentiate into myotubes on collagen type I coated plates in medium supplemented with 5% horse serum, 2 mM glutamine, and 2% penicillin/streptomycin (5000 U). COS cells were transfected using Fugene HD (Promega), according to the manufacturer's instructions and resulted in <70% of cells expressing the transfected cDNAs.

Western blot analysis

Proteins were extracted from cell lines using a lysis buffer containing: TrisHCl 50 mM, NaCl 150 mM, EDTA 2mM, Sodium orthovanadate 2 mM and TritonX100 1%. Total proteins were isolated from cell debris by centrifugation. Equal amounts of total protein (30 µg/sample) were separated on a 12% SDS-PAGE Gel and transferred onto nitrocellulose membranes. Nonspecific binding was blocked with

PBS and 0.1% Tween containing 5% skim milk for nonphosphorylated proteins and TBS 0.1% Tween containing 3-5% BSA when analysing phosphorylation state. Membranes were then incubated with primary antibodies for 1hr at RT or overnight at 4°C. After washing with PBS and 0.1% Tween or TBS 0.1% Tween (phosphorylated proteins), membranes were incubated with HRP-conjugated secondary antibodies. Repeated washing was followed by revelation using ECL detection reagents (GE Healthcare) and visualized using hyperfilm ECL or ImageQuant LAS 4000. Quantifications were performed with ImageJ software.

Constructs, inhibitors and antibodies

The constructs used in this study were pcDNA3 (Invitrogen), pcHuR. For the luciferase constructs, mouse β -AChR 3'UTR nt 1565-2151 (NM_009601.4) was amplified by RT-PCR from muscle diaphragm mRNAs, using the following primers: 5'-GGACCCTCCCGTCATTTTTG-3' and 5'-TTTTAATCATRRTTCTACATTTAATTTAATACGTG-3' with flanking SacI and XhoI restriction sites. The PCR product was digested and subcloned downstream of the Luciferase gene in pmirGlo vector (Promega). β -AChR 3'UTR ARE mutations A1720G, T1722C, and A1724G were introduced using the Quickchang IIXL site-directed mutagenesis kit (Agilent Technologies) following manufacturer instructions.

The antibodies were a mouse monoclonal anti-GAPDH clone 6C5 (Abcam), a mouse monoclonal anti-HuR 3A2 (Santa Cruz), a mouse monoclonal anti-IgG (Sigma), a rabbit polyclonal anti-phosphorylated p38 (Cell Signaling) and a rabbit polyclonal, p38 (Cell Signaling). The p38 inhibitor SB203580 (Cell signaling) used inhibits p38 α and p38 β but not p38 γ .

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TABLES AND FIGURES LEGENDS

Figure 1. ColQ regulates AChR β mRNA stability in myotubes. 5 day differentiated myotubes were treated with the transcriptional inhibitor Actinomycin D (4 μ g/ml). mRNA was isolated at different time points (0, 4, 6 and 8h) and quantified by RT-QPCR relative to 18S. AChR β mRNA decay curves are shown with all values at time 0 standardized to 100%. Values are expressed as means \pm SEM (n=3). *p<0.05; **p<0.01; unpaired Mann–Whitney’s U test.

Figure 2. HuR expression is upregulated in the absence of ColQ in myotubes. (A) microarray analysis shows that HuR mRNA levels are increased in the absence of ColQ throughout myotube differentiation in culture. (B and C), Western blot of total protein extracts show a significant increase in HuR protein levels in the absence of ColQ at T1 and T2. (B) Shows the representative western blot (C) HuR protein quantification normalized to GAPDH. Data are presented as percentage of control SEM from four independent experiments. *p<0.05; **p<0.01 unpaired Mann–Whitney’s U test.

Figure 3. HuR binds endogenous AChR β subunit mRNA in myotubes. Endogenous HuR interacts with AChR β mRNA in vitro (bottom panel). In 5 day differentiated myotubes, RNA–protein complexes were crosslinked with formaldehyde and immunoprecipitated after cell lysis using antibodies against HuR or IgG. Following crosslink reversal, the RNA was isolated from the immunoprecipitate and was used to produce cDNA by reverse transcription, followed by QPCR quantification using AChR β subunit primers. A representative western blot confirming the quantity and precipitation of HuR is shown (top panel: IN, input; FT, flow through; W, wash; E, Elution). Data are presented as percentage of control SEM from three to five independent experiments. **p<0.01; ns, not significant; unpaired Mann–Whitney’s U test.

Figure 4. HuR regulates AChR β mRNA levels via AREs located in the 3’UTR (A) Scheme of the luciferase construct containing the coding sequence of Luciferase followed by the AChR β 3’UTR sequence. The localization of the ARE motif is indicated. Below, the sequence of the ARE motif in the WT 3’UTR (WT ARE) and the mutated nucleotides in the ARE motif (Δ ARE) are shown (B) A representative western blot showing the precipitation of HuR (Top panel; IN, input; FT, flow through; W, wash; E, Elution). Quantification of Luciferase mRNA levels after RIP assays performed on COS cells transfected with WT or mutated AChR β 3’ UTR Luciferase constructs (3’UTR WT ARE and 3’UTR Δ ARE respectively) or the empty vector containing the Luciferase coding sequence without the AChR 3’UTR insert (CTRL vector) as a control (bottom panel). Samples were immunoprecipitated with either IgG as control, or with an antibody against HuR. RT–QPCR analyses revealed a significantly decreased amount of Luciferase mRNA interacting with HuR in the mutated construct. (C) Luciferase mRNA levels quantified upon the transfection of luciferase constructs containing the WT or mutated AChR β 3’ UTR in COS cells. (D) COS cells were co-transfected with reporter constructs containing the AChR β WT or mutated 3’ UTR together with pcDNA3, or pcHuR-FLAG. Luciferase mRNA was quantified by RT-QPCR and normalised to 18S. (E) Western blot against HuR

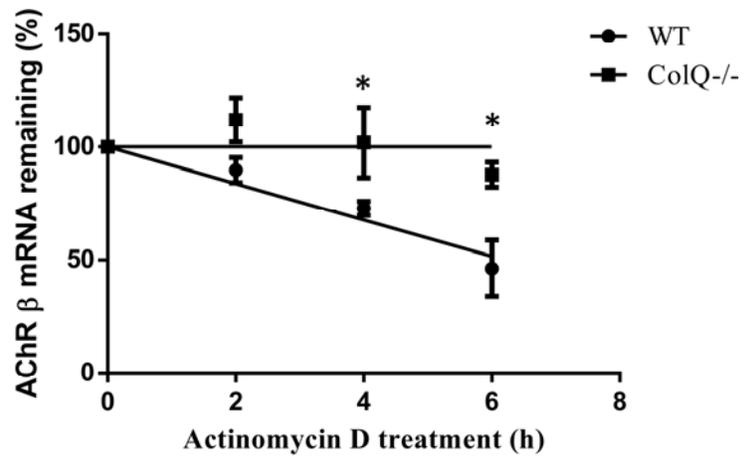
reveal endogenous HuR and transfected HuR-flag (F) Luciferase mRNA degradation assay produced by the transfection of luciferase constructs containing the WT or mutated AChR β 3' UTR in COS cells. AChR levels were determined by RT-QPCR analyses and normalised to 18S. Data are presented as percentage of control SEM from three to five independent experiments. * $p < 0.05$; ** $p < 0.01$; unpaired Mann–Whitney's U test

Figure 5. The absence of MuSK induces a decrease in HuR protein levels. (A and B) Western blot of total protein extracts show a significant decrease in HuR protein levels in the absence of MuSK. (A) Representative western blot (B) The quantitative analysis of HuR normalized to GAPDH. Data are presented as percentage of control SEM from four independent experiments. * $p < 0.05$; unpaired Mann–Whitney's U test.

Figure 6. ColQ deficiency regulates HuR expression through p38 activation in myotubes. (A and B) Western blot of total protein extracts show a significant increase in phosphorylated p38 protein levels in the absence of ColQ at T1. (A) Representative western blot (B) Quantification of phosphorylated p38 proteins normalized to GAPDH (C and D) Western blot of total protein extracts show that the treatment of muscle cells with SB203580 (10 μ M) an inhibitor of p38 induced a decrease in HuR protein levels at T1 and T2. (C) Representative western blot (D) Quantification of HuR proteins normalized to GAPDH. Data are presented as percentage of control SEM from four independent experiments. * $p < 0.05$; unpaired Mann–Whitney's U test.

ABBREVIATIONS

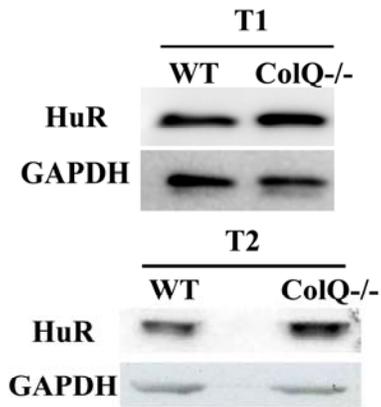
ACh	Acetylcholine
AChR	Acetylcholine receptor
ARE	AU rich element
ColQ	Collagen Q
Dok7	Docking protein 7
Elav	Embryonic lethal abnormal vision
ECM	Extracellular Matrix
GABP	GA Binding Protein
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
HuR	Human antigen R
JNK	c-jun NH2-terminal kinase
LRP4	Lipoprotein Related Protein 4
MAPK	Mitogen-Activated Protein
MyoD	Myogenic Differentiation antigen
MuSK	Muscle Specific Kinase
NF- κ B	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NMJ	Neuromuscular Junction
SMN	Survival Motor Neuron
UTR	Untranslated Region



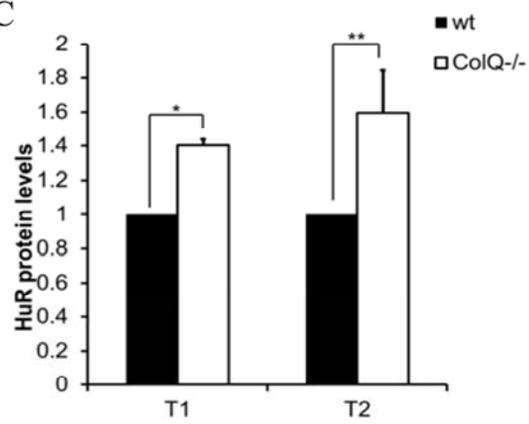
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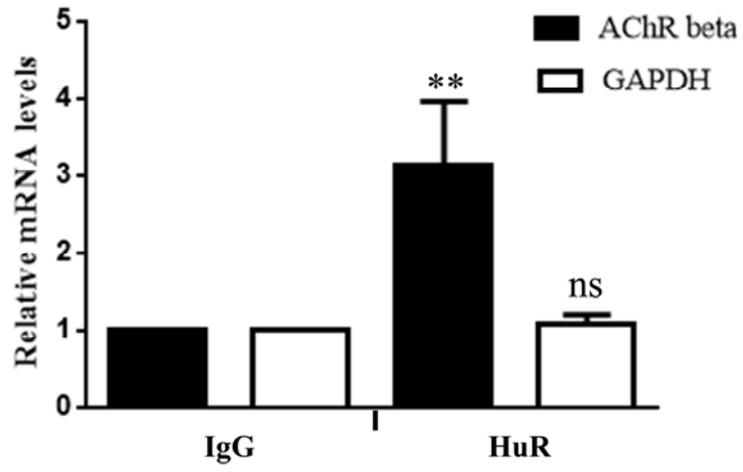
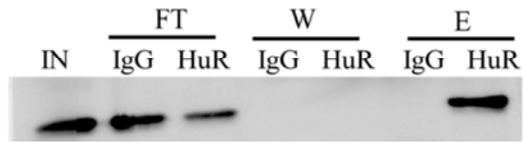
Gene(symbol)	Accession Number RefSeq	Fold change and significance		
		T1	T2	T3
		ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1 (Hu antigen R)	NM_010485	+1.85**

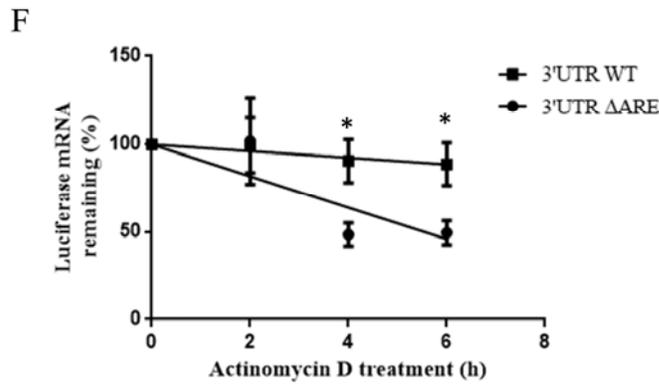
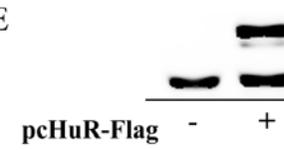
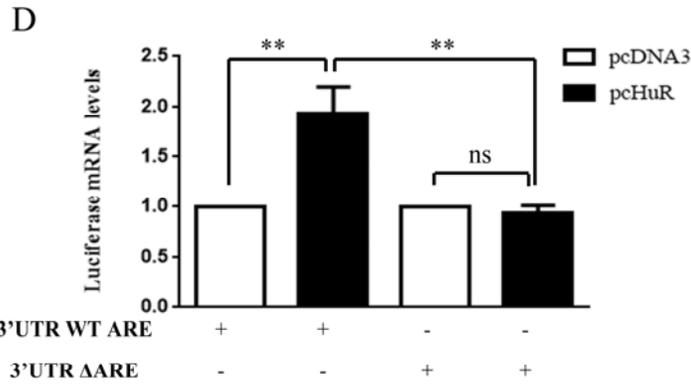
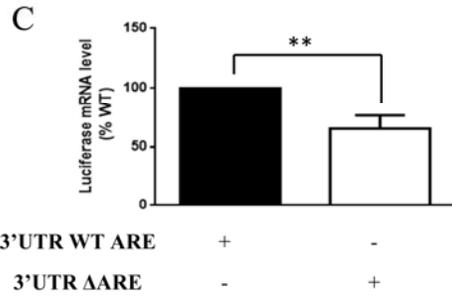
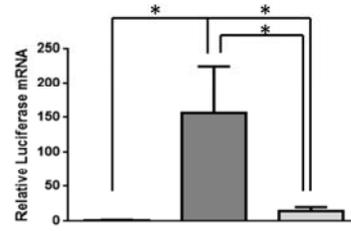
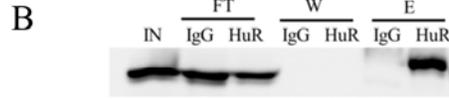
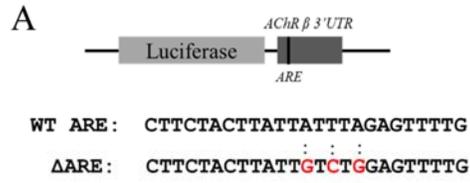
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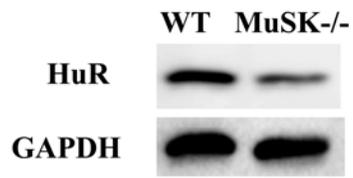
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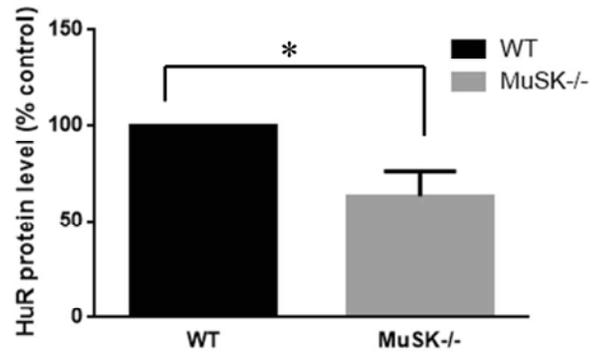


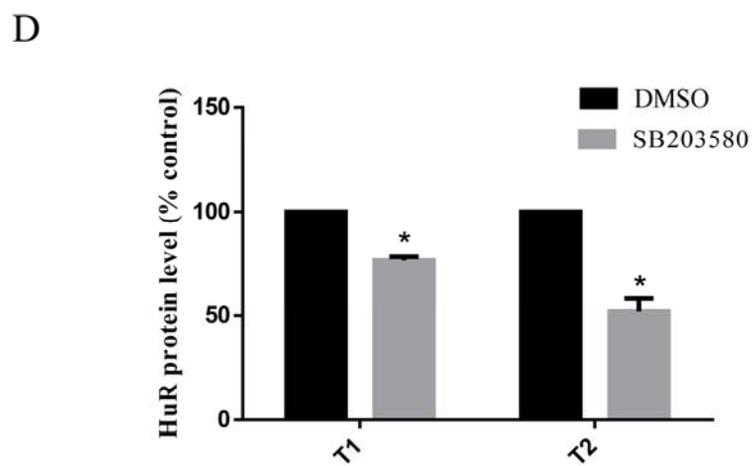
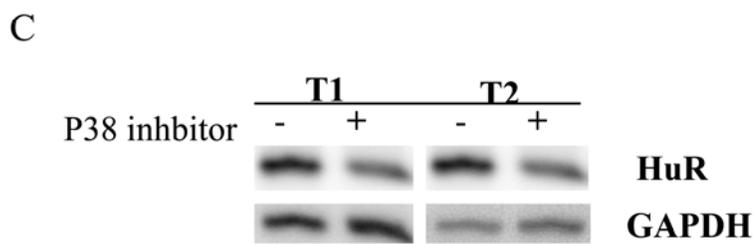
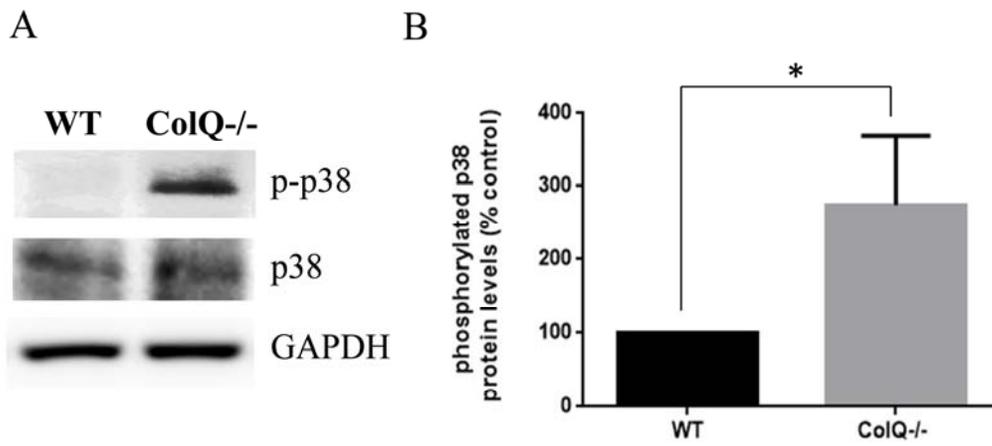


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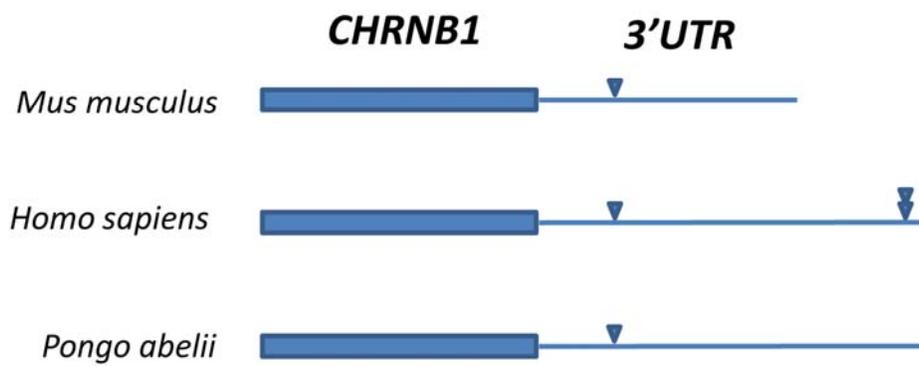
Supplemental data Figure S1. (A) 3 of the 5 AChR subunit 3'UTRs contains classic HuR binding sites. Table showing the presence of 0 to 2 ARE elements in the 3'UTR of the AChR subunits (B) Schematic representation of the AChR beta subunit 3' UTR showing the presence of conserved AREs marked by blue arrowheads. Double arrow heads indicate overlapping ARE sites.

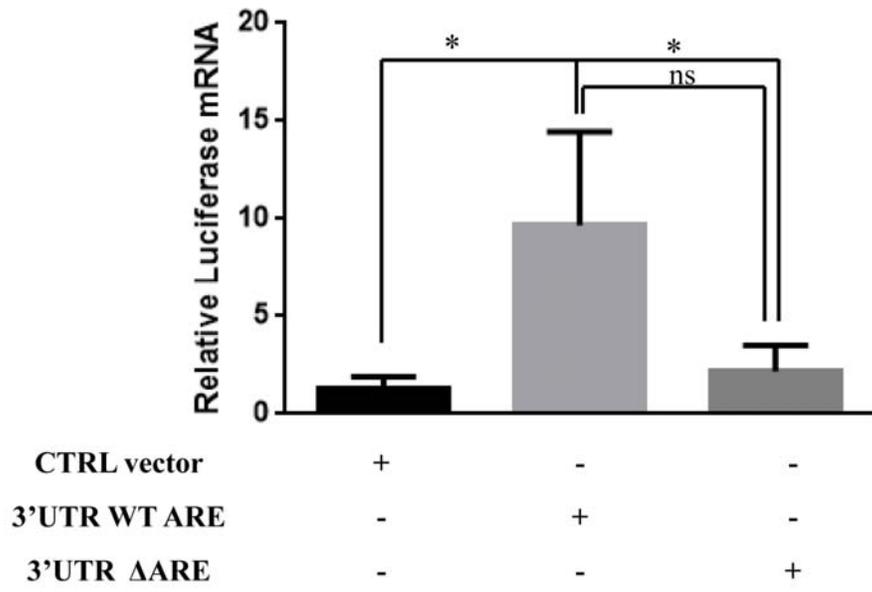
Supplemental data Figure S2. HuR interacts with the conserved ARE motif in the AChR γ 3'UTR. Luciferase mRNA levels observed after RIP assays performed on COS cells transfected with WT, mutated AChR γ 3' UTR constructs or the empty vector as a control. Samples were immunoprecipitated with either IgG as control, or with an antibody against HuR. RT-QPCR analyses revealed a significantly decreased amount of luciferase mRNA interacting with HuR in the mutated construct. A representative western blot quantifying and confirming the precipitation of HuR is shown (IN, input; FT, flow through; W, wash; E, Elution). Data are presented as percentage of control SEM from three to five independent experiments. * $p < 0.05$; ns, not significant; unpaired Mann-Whitney's U test.

A

AChR subunit type	Number of Classic ARE elements
Alpha	0
Beta	1
Delta	2
Gamma	1
Epsilon	0

B





II. PROVIDING AN UPDATED AND INTEGRATIVE VIEW OF ColQ'S FUNCTIONS.

A. Introduction

The collagen Q is a specific collagen that, until recently, had only been considered as an anchor protein for AChE at the NMJ. The importance of ColQ at the NMJ has been highlighted by the fact that over 30 mutations in the COLQ gene have been identified to be responsible for a congenital myasthenic syndrome with AChE deficiency. Moreover, our group has produced results demonstrating ColQ's signaling functions that are at least in part, mediated by the binding of ColQ to the Muscle Specific Kinase, MuSK. MuSK is a receptor for agrin and Wnts that orchestrates the different steps of neuromuscular junction formation.

In this review, we provide a brief summary of ColQ since its characterization in 1997 as an anchoring protein to acetylcholinesterase (AChE) in the synaptic cleft of the NMJ. We present the latest results on ColQ function beyond that of an anchoring protein and uncover the current state of ColQ as a signalling molecule. We discuss ColQ's regulation of AChR clustering and MuSK insertion at the NMJ post-synaptic membrane along with the expression of a number of specific synaptic genes.

The lack of AChE has been incriminated for the symptoms observed in patients along with NMJ defects in the CMS mouse model (ColQ-deficient). However, symptoms observed in the patients and mouse model of CMS are complex and AChE deficiency cannot account for all of them. Here, we compare research in CMS with AChE deficiency patients and COLQ knock-out mouse models of CMS to provide evidence into the possibility that defects observed in synaptic congenital myasthenic syndromes might be linked, at least in part, to alterations in ColQ signaling.

Finally, we discuss future research directions to understand how ColQ may modulate the action of the other ligands of the MuSK/LRP4 complex, in cooperation, to coordinate the

different steps of NMJ formation and maintenance. In this context, it's worth mentioning that preliminary experiments show a defect in NMJ formation during embryonic development (Figure 27).

The components of the MuSK receptor complex are expressed by muscle cells before innervation and represent the core system from which the NMJ is formed and maintained. Thus, we must now decipher how the same receptor complex is involved in multiple developmental processes.

The diversity in MuSK function is probably dependent on its three identified direct or indirect ligands; agrin, ColQ and Wnts (Wnt4 and Wnt11). Wnt4 and Wnt11 play an early role in synapse formation, while agrin acts once the contact between nerve and muscle is established. These three ligands of the MuSK/LRP4 complex are expressed during synapse formation and maintenance, with each ligand having a selective developmental role. We must now determine how these ligands activate their respective function through the interaction with the same MuSK complex. However, it is possible that cross-talk between these ligands occurs and in this context, a particular focus will be placed on the Wnt/ColQ functional interaction since they both bind MuSK.

Thus, we would like to assess how ColQ affects the activity of the two other ligands. In order to determine these unknowns we must:

- 1) Decipher the binding sites of the ColQ on MuSK/LRP4 (Wnt and Agrin binding sites have already been determined). This last result will be crucial to understand the functional relationship between ColQ and the two other ligands. Since both ColQ and Wnts are binding MuSK, one could ask if ColQ influences the strength of Wnt binding. More, could ColQ modify the conformation of the MuSK-LRP4 complex and therefore affect the binding of Agrin to LRP4 in the presence of ColQ.

- 2) The geometry of the macro-complex formed by the ligands and the receptors to identify the signaling pathways induced by each of the ligands.
- 3) The cross-talk between these putative pathways, or the control by some of the ligands on a main pathway. Is ColQ regulating MuSK phosphorylation? Are the ligands of MuSK acting on similar or different phosphorylation sites?

Neurofilament/ Synaptophysin

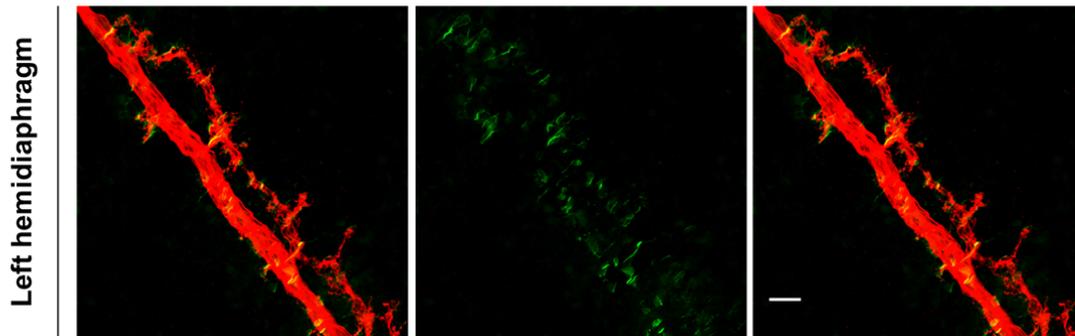
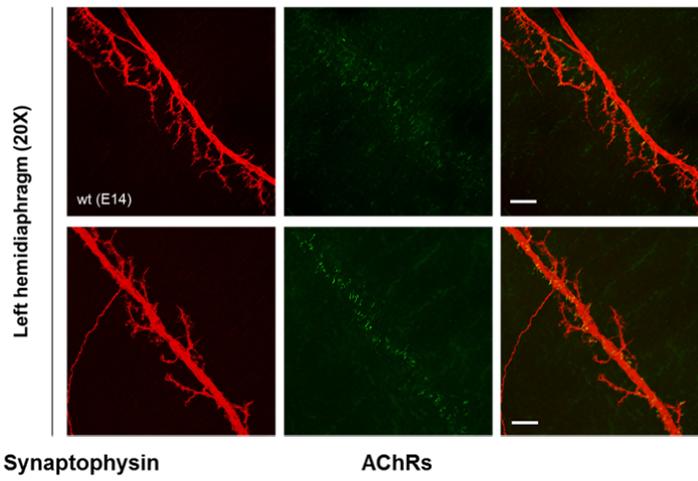
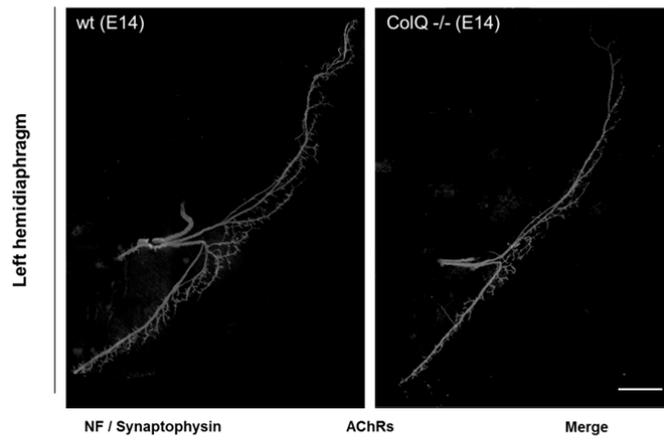


Figure 27. Synaptic phenotype of ColQ mutant diaphragm at E14 **A.** Confocal images (10X) of whole-mount diaphragm muscles from stage E14 control littermates (WT) or ColQ^{-/-} stained with neurofilament and synaptophysin. A representative left hemidiaphragm of each phenotype is shown. **B.** Confocal images (20X) of whole-mount diaphragm muscles from stage E14 control littermates (WT) or ColQ^{-/-} stained with neurofilament, synaptophysin and α -bungarotoxin. A representative left hemidiaphragm of each phenotype is shown. **C.** Confocal images (20x crop) of whole-mount diaphragm muscles from stage E14 ColQ^{-/-} stained with neurofilament, synaptophysin and α -bungarotoxin. A representative left hemidiaphragm is shown. Scale bar : 200 μ m.

ARTICLE 2

GENERAL DISCUSSION

The proteins of the synaptic ECM are crucial for the signal transduction processes that develop and maintain the NMJ. ColQ, a protein of the synaptic ECM is the causative gene in CMS with AChE deficiency; however nothing was known about ColQ beyond that of an anchoring protein for AChE. The absence of ColQ or its low expression induces an increase in ACh in the synaptic cleft a consequence of the lack of AChE clusters. Recently, our group showed that the loss of ColQ leads to muscle intracellular modifications of synaptic protein levels revealing for the first time the possibility that ColQ induces a signalling pathway. The most striking effect of the absence of ColQ is an upregulation of all AChR subunit mRNAs correlated by an increase in their protein levels (Sigoillot et al., 2010), a process that may be interpreted as an adaptation to the overload of ACh in the synaptic cleft at least partially. Here, we show that a post-transcriptional mechanism of AChR mRNA stabilization is responsible for the increased levels of AChR mRNAs. In addition, the absence of ColQ increased the mRNA and protein levels of HuR. Furthermore, we have shown that HuR is capable of binding AChR subunit mRNA and increasing the stability of subunit mRNA. We also provide evidences that ColQ regulates HuR mediated AChR subunit stability through MuSK and that the p38 signaling pathway controls the levels of HuR in a ColQ dependent manner. A diagram of our proposed model is described in figure 28.

Moreover, we have provided insight into the phenotypic effect of the loss ColQ in CMS. We show that the absence of ColQ affects the synaptic ECM.

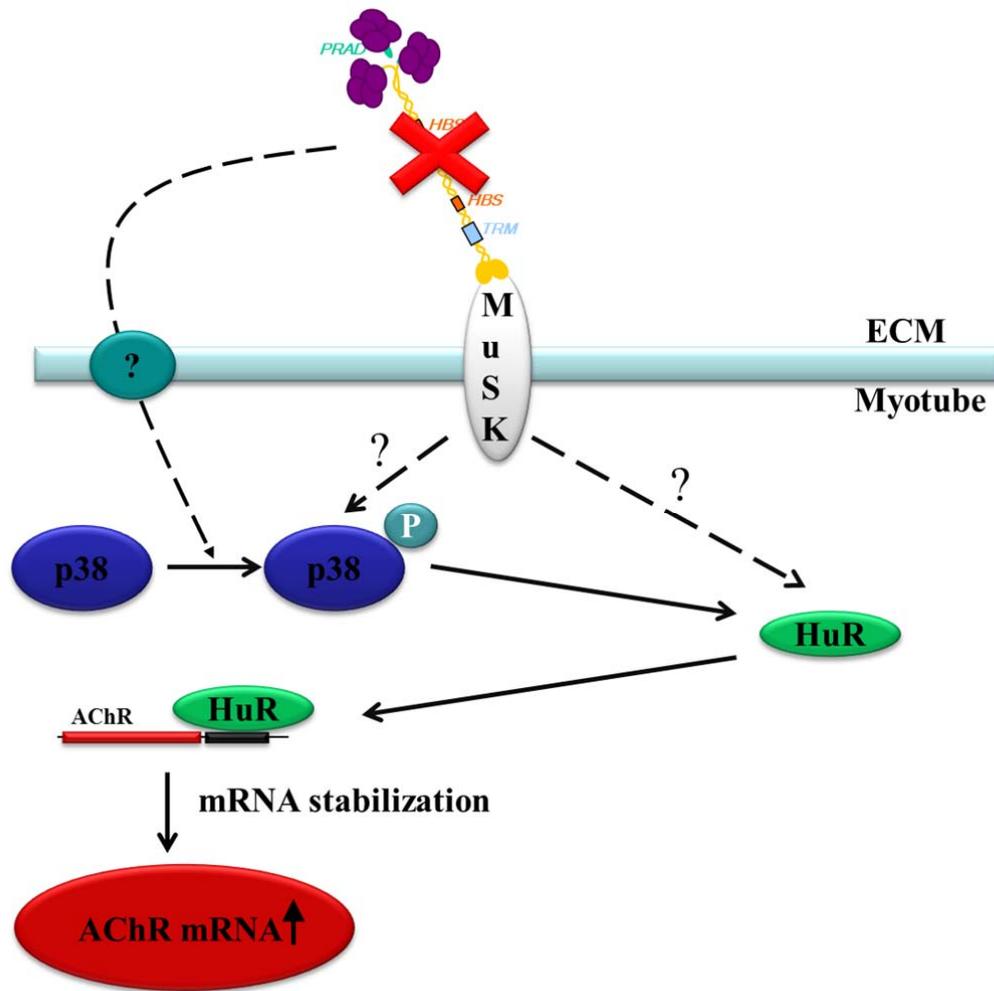


Figure 28. Proposed model for the ColQ dependent regulation of AChR subunit mRNA. The absence of ColQ increases HuR expression. HuR is able to interact with the 3'-UTR of AChR subunit transcript. This binding stabilizes AChR mRNA which results in an increase in AChR steady-state mRNA levels. We propose that ColQ regulates HuR mediated AChR subunit stability through MuSK and that the p38 signaling pathway controls the level of HuR in a ColQ dependent manner. Dashed lines represent what has yet to be confirmed. It should also be noted that solid arrows do not represent direct interaction

I. FURTHER DECIPHERING THE CASCADE LINKING ColQ TO AChR mRNA LEVELS

A. How is ColQ regulating p38 phosphorylation?

In search for a signalling pathway induced by the absence of ColQ, we reveal that the absence of ColQ increases the phosphorylation and thus the activation of p38. As previously described (pg.80), p38 activity is dependent on the phosphorylation of its active loop by MKKs/MAP2Ks. It is possible that the ColQ dependent upregulation of active p38 is caused by an increase in its phosphorylation, however the absence of ColQ could also be inhibiting the dephosphorylation of p38 which would also result in an increase in the phosphorylated p38 pool.

The dephosphorylation/deactivation of p38 is regulated by phosphatases that dephosphorylate serine/threonine residues located in the active loop of the p38 isoforms. These phosphatases include general phosphatases such as protein phosphatase 2A (PP2A) and PP2C or dual specificity phosphatases/MAPK phosphatases (DUSPs/MKPs). MKPs increase the phosphatase activity of DUSPs. They also provide a docking domain for DUSPs and a domain for the MAPK substrate. MKP 1, 4, 5, and 7 have been shown to dephosphorylate p38 α and β (Cuadrado and Nebreda, 2010; Owens and Keyse, 2007). Therefore, in order to determine how ColQ is regulating p38 activity, both kinases and phosphatases should be assessed.

B. Activation of p38, a stress response?

It is known that p38 α phosphorylates HuR inducing its translocation to the cytoplasm where it interacts with its target mRNA (Lafarga et al., 2009). In addition, the p38 α isoform has been found to activate NF- κ B (Olson et al., 2007). Thus p38 α appears to be a good candidate for ColQ dependent HuR regulation as it is capable of regulating HuR expression and localization. Furthermore, *in vitro* experiments show that the transcriptional regulation of

Rapsyn by NF- κ B was not altered by agrin. Our team has shown that ColQ increases Rapsyn mRNA levels and also alters the clustering of AChR at the post-synaptic membrane in the absence of agrin. Therefore, it is possible that ColQ could be the signalling molecule that influences MuSK to activate NF- κ B induced transcription.

C. What is upstream of p38 activation?

1. The MuSK signaling platform

We have previously demonstrated that ColQ dependent regulation of AChR subunit mRNA relies on its interaction with MuSK (Sigoillot et al., 2010a). Along the same lines our data shows ColQ's inhibitory effect on HuR expression could be mediated by MuSK. The transcription of HuR is regulated by the transcription factor NF- κ B and by Smads (Jeyaraj et al., 2010; Kang et al., 2008). Thus, the possibility that ColQ by interacting with MuSK recruits one of these transcriptional molecules remains to be investigated. Interestingly, NF- κ B which is enriched around AChR aggregates and in nuclei of differentiated myotubes has been shown to increase AChR clustering and Rapsyn mRNA levels by a transcriptional mechanism (Wang et al., 2010).

Conversely to what is seen *in vitro*, the *in vivo* data in P30 gastrocnemius muscle does not show an upregulation of HuR protein in the absence of ColQ however the upregulation in AChR subunit mRNA is seen both *in vitro* and *in vivo*. This apparent discrepancy could be explained as follows, HuR is a ubiquitous protein that is highly concentrated in the nucleus of myofibers. HuR shuttles between the nucleus and cytoplasm where it serve its function as an RNA binding protein. Primarily, HuR is translocated to the cytoplasm upon stimuli such as apoptosis, cell division, and myogenesis (von Roretz et al., 2011). The phosphorylation state of HuR plays a large role in HuR localization. Phosphorylation of HuR at different residues by multiple kinases alters not only the subcellular localization of HuR but concomitantly

interacts with target mRNAs. (Srikantan and Gorospe, 2012). For example, the phosphorylation of HuR in its nucleocytoplasmic shuttling domain (HNS) by Cdk1 induces its interaction with 14-3-3 θ resulting in the nuclear retention of HuR (Kim et al., 2008a). Thus it is possible that *in vivo*, MuSK may alter the phosphorylation state of HuR.

2. The disruption of the ECM

Why is the p38 pathway activated in absence of ColQ? It is possible that the extracellular matrix is disorganized, a trait that is suggested by the electron micrographs of the NMJ deficient for ColQ with Schwann cells processes intruding into the synaptic cleft (Feng et al., 1999). We have also shown that ColQ alters the expression of its partners perlecan and α Dystroglycan, two key components of the ECM. Together, these modifications may induce a cellular stress and because the p38 pathway has been reported as a major stress response signaling pathway, we hypothesise that ColQ would be an indirect effector of this signaling pathway.

i. MuSK acts as an “ECM sensor”?

The stress response could be mediated by MuSK or other post-synaptic transmembrane receptors such as integrins or DG. Here, it should be pointed out that several components of the ECM such as perlecan and biglycan are indirectly and directly binding MuSK (Amenta et al., 2012; Brandan et al., 1985) and thus MuSK could act as a “sensor” of ECM integrity. In this context, it is interesting to note that the lack of ColQ or biglycan both lead reduced levels of membrane MuSK highlighting the role of ECM molecules in the stabilization of MuSK at the membrane (Amenta et al., 2012; Sigoillot et al., 2010a).

Besides MuSK, two other receptors could activate p38. Integrins are postsynaptic transmembrane receptors which are major sensor of mechanical forces in muscle. The α 7 β 1 integrin is enriched at the post-synaptic membrane of the neuromuscular junction and is

upregulated in patients with Duchenne muscular dystrophy and mdx mice where the attachment of the skeletal muscle to the extracellular matrix is altered (Hodges et al., 1997; Kaariainen et al., 2002). However, $\alpha7\beta1$ integrin levels are not significantly affected either in vitro or in vivo (S. Sigoillot, et al., in preparation) suggesting that integrins might not be involved in the p38 activation in response to ColQ deficiency.

Furthermore, ColQ deficiency regulates α Dystroglycan mRNA and its post-translational glycosylation altering its function *in vitro*. α Dystroglycan connects the ECM to the intracellular cytoskeleton of the muscle and thus could provide the link for the internalization of ColQ signalling (Henry and Campbell, 1996). Thus it would be interesting to determine if changes in α Dystroglycan protein levels could activate the p38 pathway.

ii. Partial denervation

The loss of the motor neuron (denervation) has been the root cause of such neuromuscular disease as Amyotrophic lateral sclerosis (ALS) and spinal muscle atrophy (SMA). We were interested in determining if ColQ was showing a similar phenotype to denervated muscle. Microarray studies performed by our team *in vitro* and verified *in vivo* have provided insights into the molecular targets of ColQ. It was brought to our attention that a number of synaptic genes upregulated in denervated muscle are also upregulated in ColQ-deficient muscle such as those of the AChR subunits (Goldman et al., 1988; Witzemann et al., 1987; Witzemann et al., 1991). In denervated muscle, an increase in the levels of of ACh subunits α , β , γ , δ , but not ϵ is observed resulting from the transcriptional activation of the corresponding genes by myogenin (Kostrominova et al., 2000; Witzemann et al., 1991). This factor is a member of the myogenic transcription factors that binds the E-box, a cis element found in the promotor of most synaptic genes (Murre et al., 1989). We have yet to determine the transcriptional impact that the absence of ColQ has on these synaptic genes, rather our focus has been placed on their post-transcriptional regulation. However, the myogenic factors regulating synaptic

gene expression such as AChR are only increased *in vitro* in the absence of ColQ while synaptic gene expression is increased both *in vitro* and *in vivo*.

Another common phenotype of denervation is the reappearance of the embryonic AChR γ subunit and its scattering along the entire myofiber. AChR ϵ subunit however is unaffected by denervation and continues to be expressed and localised at the post-synaptic membrane (Witzemann et al., 1991). ColQ deficient NMJs show a different scenario; both the γ and ϵ subunits have an increase in their mRNA levels with preliminary results suggesting that the AChR γ subunit is localised only to the post-synaptic muscle membrane. Therefore, ColQ deficient muscles appears to mimic only some characteristics of denervation indicating the abundance of neuronal factors such as ACh does not fully explain phenotype of CMS with ColQ deficiency. It remains that the invasion of Schwann cell in the synaptic cleft induced by the absence of ColQ could result in a partial denervation (Feng et al., 1999).

II. THE MOLECULAR SIGNATURE OF CMS WITH AChE DEFICIENCY: SPECIFIC AND COMMON TRAITS WITH OTHER CMS, SIGNIFICANCE FOR THERAPEUTIC APPROACH.

A. ECM gene mutations

Currently, other than ColQ, only two genes of the synaptic basal lamina have been found to hold mutations that cause CMS in human: Agrin and Laminin β 2. Mutants for all agrin isoforms die shortly after birth while the laminin β 2 mutants die two to four weeks postnatal (Maselli et al., 2012). Although mutations in these synaptic genes vary in severity, they do have a common phenotype, the encasement of the nerve terminal by Schwann cells (Maselli et al., 2012). The invading of Schwann cells in the synaptic cleft prevents the close contact between the pre and post synaptic cells.

Hypomorphic mutations in the gene of another basal membrane protein perlecan cause Schwartz-Jampel syndrome (SJS) a form of congenital peripheral nerve hyperexcitability (PNH). SJS is characterized by continuous spontaneous muscle activity. Hypomorphic mutations in the perlecan gene lead to low protein levels of extracellular perlecan in SJS patients and model mice (Stum et al., 2005; Stum et al., 2006). Interestingly, the loss of extracellular perlecan results in partial endplate AChE deficiency and like the mutants described above, causes abnormalities in myelination (Bangratz et al., 2012; Stum et al., 2008).

Therefore, it would be interesting to explore the activity of the p38-HuR pathway in these mutants to see if our stress hypothesis holds true in other diseases of the NMJ.

B. Therapeutic strategies

Ephedrine and salbutamol, two adrenergic agonists, have been found to be beneficial in treating CMS patients with AChE, Dok-7, and β (2)-laminin deficiencies and also SMA patients (Kinali et al., 2002; Liewluck et al., 2011). Furthermore, salbutamol has been found

to increase SMN mRNA levels (Angelozzi et al., 2008). Based on the phenotypic similarities between these neuromuscular diseases it would be worth determine if Ephedrine or salbutamol alter the activity of the p38 α / β pathways and HuR.

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**ANNEX A: IDENTIFYING CoIQ'S PHENOTYPIC
AND MOLECULAR EFFECTS IN A MODEL OF
CMS WITH AChE DEFICIENCY**

A. Introduction

As previously mentioned, congenital myasthenic syndrome (CMS) is a class of inherited diseases linked to mutations in synaptic genes that affect the neuromuscular transmission. CMS with AChE deficiency is a class of CMS caused by the absence or the poor expression of acetylcholinesterase (AChE). However, the syndrome is not due to mutations in the AChE gene but to mutations in the gene of its anchoring protein, COLQ. Furthermore, a mouse model of CMS with AChE deficiency has been generated by the knock-out of COLQ (Feng et al., 1999) with the mouse portraying most of the characteristic features observed in patients suffering from CMS with AChE deficiency (Eymard et al., 2013). Interestingly, restoring ColQ in these mutant mice restores a normal phenotype in the motor functions, synaptic transmission and structure of the NMJ (Ito et al., 2012).

The Collagen ColQ, plays a critical role at the mammalian NMJ synapse through the anchoring of AChE in the synaptic basal lamina (Krejci et al., 1999; Krejci et al., 1997). Recently, based mostly on a microarray study, we have demonstrated (Sigoillot et al., 2010a) a functional role of ColQ, through the regulation of AChR cluster density and synaptic gene expression via its interaction with MuSK.

The new functional role of ColQ at the NMJ and its validity in the CMS with AChE deficiency mouse model captivated us to determine the molecular and phenotypic hallmarks of CMS with AChE deficiency in a mouse model and explore the role of ColQ as more than a structural protein.

B. Primary Results

Here, we have conducted a large genetic screen by microarray to identify the molecular targets of ColQ and revealed molecular defects that could explain the observed phenotype in the mouse model of CMS with AChE deficiency. To define the network of genes under the control of ColQ during muscle cell differentiation, we aimed to compare the transcriptome of WT and ColQ-deficient muscle cell lines produced from the WT and ColQ knock-out mice, at the three time points of myotubes differentiation, labeled as T1, T2 and T3.

The results obtained *in vitro* show that the absence of ColQ causes modification of 38% of specific mRNA levels assessed, in some instances occurring only at the beginning (T1) or the end of the muscular differentiation (T3), while in others, modifications occur at all stages of differentiation (T1, T2, and T3).

For a subset of mRNA, the study was extended *in vivo* using the Taqman Low-Density Array (TLDA) technology which allowed us to quantify the levels of about one hundred specific mRNA from the same sample. Gastrocnemius muscle was used for this *in vivo* study at P7 and P30.

Comparison of *in vitro* and *in vivo* results revealed that a large number of mRNAs which are downregulated *in vitro* are upregulated *in vivo*, indicating that a strong mechanism of compensation occurs *in vivo*. A second major observation is that mRNA coding for contractile proteins or muscle structural proteins are not affected by the absence of ColQ. These results corroborate previous results using electron microscopy showing a normal structure of the muscle in the ColQ knock-out mice. In addition, using Gene Oncology (GO) a bioinformatics analysis of data, it appears that most of the mRNA significantly affected by the lack of ColQ fall in a “skeletal and muscular system development and function”.

Initially, this study was motivated by the structural defects observed in ColQ deficient mice. As shown by electron microscopy, Schwann cells extend processes in the synaptic cleft in a

number of NMJs (Feng et al., 1999). This can be interpreted by a disorganization of the ECM. ColQ is a protein belonging to the extracellular matrix (ECM). Its central collagen-like domain binds directly ECM proteins perlecan, and indirectly through perlecan to the transmembrane protein dystroglycan (DG). Thus, we set out to determine if ColQ's absence leads to a disruption of the ECM and muscle cytoskeleton through DG which connects the ECM to the cytoskeleton. Studies *in vitro* showed a significant decrease in perlecan at T2 and dystroglycan mRNA levels in the absence of ColQ. Interestingly *in vivo* results showed an inverse result with perlecan mRNA levels showing a significant upregulation at P30 while dystroglycan mRNA levels remained unchanged.

Dystroglycans function as an ECM receptor requires its glycosylation (Martin, 2003). Glycosylated DG were decreased by 20% in the absence of ColQ at T2 and T3 compared to control condition (Article 3: Figure 3). Because the glycosylation of DG is crucial for laminin binding, it would be worth determining in this context the localization of laminin at the NMJ in the absence of ColQ.

Furthermore, DG is expressed all along the muscle fibers but is preferentially accumulated at the NMJ with AChRs, we also analyzed the localization of active form of DG in myotubes forming AChR clusters using an antibody that recognizes the glycosylated form of DG. Immunofluorescence experiments performed at T2 revealed that DG is co-clustered with AChRs in WT myotubes whereas ColQ^{-/-} myotubes showed some AChRs devoid of glycosylated DG (Article 3: Figure 3C). Together, these results suggest that ColQ might regulate DG functional expression and localization, thus potentially controlling the molecular cascades linking ECM to cytoskeleton.

Since DG mRNA levels or post-transcriptional modifications are altered in the absence of ColQ and dystroglycan binds directly to dystrophin (Ervasti and Campbell, 1993; Ibraghimov-Beskrovnaya et al., 1992), we explored signs of Dystroglycan-dystrophin

associated functional defects. The disruption of the ECM and cytoskeleton via the dystrophin complex is often associated with diseases like dystrophy/myopathy, thus we determined whether the modifications of ECM gene expression detected in ColQ^{-/-} myotubes were also observed in muscle from ColQ^{-/-} mice. RT-QPCR was performed in mice at P7 and P30 to analyse ECM genes encoding structural proteins, proteins involved in ECM regulation and the dystrophin complex. This assay revealed very few significant variations. However, a number of mRNA are upregulated, in contrast to what is observed *in vitro*, promoting the idea that compensation mechanisms and molecular dynamics are taking place after birth.

To gain insight into how these molecular changes affect the muscle phenotype of the CMS with AChE deficiency mouse model, we conducted a study of morphological dystrophic features on fast and slow muscle of the ColQ deficient mice 7, 30 and 90 days after birth. ColQ^{-/-} mice were found to be smaller in size than the WT from the same litter, a difference that worsens with age. Here it should be mentioned that ColQ-deficient and WT embryos present no differences in weight. Muscles and muscle fibers from ColQ-deficient mice are smaller in diameter and muscles contain fewer muscle fibers.

Muscle dystrophy is thought to be mostly characterized by: 1) an increase in the presence of slow fibers and 2) an increase of necrotic fibers and or regenerating fibers. Fiber analysis of ColQ deficient mice at P30 revealed no increase in the amount of slow type fibers in the sternomastoid (STM; fast muscle) but showed a 50% decrease in the soleus (Sol; slow muscle). In addition, small increases in the amount of necrotic fibers or regenerating fibers were seen in both the slow and fast muscle fibers. In conclusion, the ColQ^{-/-} mice seems to be mildly affected by dystrophic phenomena.

C. Conclusions and Perspectives

Our data suggest that the absence of ColQ could lead to the disorganization of the ECM and of its linkage to the muscle membrane via its interaction with dystroglycan. However these effects are less dramatic, or in some cases even opposite *in vivo* and are likely due to a compensation mechanism.

Moreover, the CMS with AChE deficiency mouse model exhibits phenotypical signs of general atrophy since body weight, muscle diameter, fiber number per muscle, and diameter of each muscle fiber were decreased. However, some of these parameters were more specifically affected in sternomastoid fast muscle while soleus muscle presented a more specific muscle atrophy.

ARTICLE 3

Title: Molecular and phenotypical analysis of ColQ-deficient muscles in a model of myasthenic syndrome

Short Title: Gene expression of ColQ-deficient muscles

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Key Words: Gene profiling, Muscle atrophy, MuSK, Dystroglycan.

INTRODUCTION

Congenital myasthenic syndromes (CMS) correspond to a class of human pathologies resulting from mutations in genes expressed at the neuromuscular junction (NMJ). These disorders are inherited in an autosomal recessive fashion. They are characterized by a dysfunction of the NMJ leading to muscle weakness and excess fatigability together with additional specific symptoms related to the mutated gene and its function (1, 2). CMS are usually classified according to the normal location of the gene product. Three types of CMS cover the functional domains of the synapse with presynaptic, synaptic and postsynaptic CMS. One of the synaptic CMS is caused by the absence or the poor expression of acetylcholinesterase (AChE) in the synaptic basal lamina. The first symptoms are detected in the neonatal period or the childhood and most of the patients are severely disabled. For the majority of the patients, the clinical picture includes muscle hypotonia, ptosis, respiratory insufficiency and delayed motor development (3). The diagnosis is based on the presence of repetitive compound muscle action potential (CMAP) after single stimulation, slow response of the pupil to light and absence of AChE visualization. The syndrome is not due to mutations in the AChE gene but to mutations in COLQ gene, a gene that encodes a specific collagen and anchors AChE in NMJ basal lamina (4). About 30 mutations have been identified in this gene to date and are spread all along the gene in different functional domain (5). The major form of AChE expressed at the NMJ is a heteromeric-complex containing a variant of AChE (AChE_T) linked to ColQ. In this form so-called asymmetric form in the past, a triple helix of ColQ binds one to three tetramers of AChE, the most saturated form of ColQ being present at the NMJ. ColQ is strictly expressed at the NMJ in fast muscles but is also present although in lower amount in extrasynaptic domains in a slow muscle such as the soleus (6). ColQ contains four functional domains including the N-terminus that binds an AChE tetramer, the collagen domain, the proximal C-terminus domain responsible for the formation of the triple helix and the distal C-terminus domain involved in an interaction with MuSK, a muscle-specific tyrosine kinase receptor (7-9). The collagen domain contains two heparin binding sites that are presumed to be responsible for the interaction between perlecan, a heparan sulfate proteoglycan

and ColQ (10, 11). Moreover, perlecan binds dystroglycan (DG), a transmembrane protein that plays a major role in linking the extracellular matrix to the cytoskeleton (12). Therefore, as in ColQ mutant, AChE is absent from NMJs of perlecan and DG deficient mice (Arikawa-Irosawa et al., Perlecan null; Jacobson et al., DG null).

A mouse model of CMS has been generated by invalidation of the COLQ gene (13). As expected, the NMJ of these mice are devoid of AChE and mice exhibit myasthenic syndromes. Patients with mutations in perlecan present a Schwartz-Jampel syndrome (SJS) characterized by myotonia (14-16). Interestingly, although SJS model mice expressed very low levels of perlecan and AChE, these mice exhibit a clearly different phenotype compared to ColQ null mice suggesting specific roles for perlecan and ColQ at NMJ (Stum et al., 2008).

As recently shown, besides anchoring AChE, ColQ participates in postsynaptic differentiation by regulating the density of AChR and the size of the synaptic clusters, a process which is mediated by ColQ interaction with MuSK (17). Here, we have conducted a large genetic screen to identify the molecular targets of ColQ and revealed molecular defects that could explain the observed phenotype in the mice model of CMS. We have extended our molecular study to the analysis of muscle type fiber phenotype. The results point to two main consequences of the deficit in ColQ, a synaptic atrophy that could be linked to the MuSK pathway and an upregulation of a subset of synaptic genes.

MATERIALS AND METHODS

Antibodies and reagents

α -bungarotoxin (α -BTX) Alexa Fluor[®] 594 conjugate, Alexa Fluor[®] 488 or 594 conjugated goat anti-rabbit or anti-mouse were purchased from Invitrogen Molecular Probes. Mouse monoclonal anti-GAPDH antibody (clone 6C5) was purchased from Abcam. I1H6C4 (western blot) and VIA4 (immunofluorescence) monoclonal antibodies to α -DG were purchased from Upstate. Myosin Heavy Chain (MHC) Slow (NCL-MHCs) was obtained from Novocastra. Lectin-FITC was purchased from Sigma-Aldrich. DAPI was purchased from Euromedex. Horseradish Peroxidase-conjugated sheep anti-mouse IgG polyclonal antibody was obtained from GE Healthcare-Amersham.

Animals

Generation of ColQ knock-out mice was described in Feng et al. (13). The use of animals is in accordance with the protocol approved by local ethics committees. Standard breeding methods were used to generate mutants.

Muscle cell culture

The two muscle cell lines, MLCL wild type (wt) and MLCL ColQ-deficient (ColQ^{-/-}) were generated as previously described (9). Myoblasts were cultured on plates coated with collagen Type I (Iwaki, Japan) maintained in DMEM supplemented with 10% fetal bovine serum, 20% horse serum, 2 mM glutamine, 2% penicillin/streptomycin (5,000 U) and 20 U/ml of γ -interferon (Roche Diagnostics; Mannheim, Germany) at 33°C in 8% CO₂. All reagents for the culture medium were purchased from Invitrogen. Cells were differentiated into myotubes in the same medium with 5% horse serum and without γ -interferon (differentiation medium). The commercial collagen-coated plates provide a minimal matrix for the cell culture and allow reproducibility between experiments.

Three stages of muscle cell differentiation were selected for analysis: T1 when cells are mostly myotubes (day 0), T2 when AChR clusters are visualized (day 2) and T3 when both AChR and acetylcholinesterase (AChE) clusters are observed in wt but not in ColQ^{-/-} myotubes (day 5)(18).

Western blot analysis

Proteins were extracted from cells using lysis buffer (TrisHCl 50mM, NaCl 150mM, EDTA 2mM, Sodium orthovanadate 2mM and TritonX100 1%). Equal amounts of proteins (30 µg/sample) were first separated by a 7% NuPAGE Novex Tris-Acetate Gels according to Invitrogen protocols, then electrotransferred to nitrocellulose membranes (TransBlot[®] Transfer Medium, Bio-Rad) (19). Membranes were blocked in PBS supplemented with Tween 0.1% (PBST) and non-fat milk 5% and incubated with various antibodies in PBST-milk 5%. After washing in PBST, membranes were incubated with Horseradish Peroxidase-conjugated secondary antibodies in PBST-milk 5%. Bound antibodies were revealed using ECL plus detection reagents (GE Healthcare-Amersham) and visualized with hyperfilm ECL. Protein loading was controlled by probing with anti-GAPDH antibody. Results obtained were quantified using ImageJ (version 1.37c) software.

Immunofluorescence

Myotubes immunostaining was performed after fixation in 2% PFA-phosphate buffer (PBS). Before incubation with antibodies, non-specific binding was blocked using DakoCytomation Protein block (Glostrup, Denmark) for 5 min. Cells were first incubated with the primary antibody for 1 h at RT, then washed in Glycine-BSA (0.15%:0.5%) buffer and incubated with a fluorochrome-conjugated secondary antibody for 30 min. Slides were mounted in Vectashield Hard Set Mounting Medium for fluorescence (Vector Laboratories).

For sections analyses, dissected sternomastoid fast-type muscles and soleus slow-type muscles from 3 to 4 thirty days postnatal (P30) wt or ColQ^{-/-} mice were fixed in 3% PFA-PBS buffer for 1 hour at 4°C, rinsed twice at 4°C in PBS, cryoprotected by successive incubations in 5%, 10% and 25% sucrose-PBS buffer at 4°C, and embedded in TissueTek (Sakura) before freezing in isopentane cooled with liquid nitrogen. 14 µm cryostat cross sections were labelled as same as for myotubes immunostaining.

All images were collected on a microscope (model BX61; Olympus) equipped with a Fast 1394 Digital CCD FireWire camera (model Retiga 2000R; Qimaging) and a 20X objective or a 40X oil objective (numerical apertures: 0.50 and 1.0, respectively; Olympus). Image capture was made using ImageProPlus software (version 5.1). The diameters of muscles or muscle fibers were measured using ImageProPlus software. Quantifications of the number of fibers per muscle or with central nuclei were realised using ImageJ (version 1.37c) software.

RNA preparation

Total RNA from MLCL wt and ColQ^{-/-} muscle cells were extracted at T1, T2 and T3 time points by using the RNeasy Protect Mini-Kit (Qiagen; Germantown, MD) and by performing DNA digestion according to the manufacturer's instructions. RNA quality was assessed by polyacrylamide-gel microelectrophoresis (Bioanalyser 2100, Agilent Technologies; Massy, France) and RNA concentration was measured by spectrophotometry (Nanodrop). Lack of genomic DNA contamination was verified by PCR. Total gastrocnemius fast- and slow-muscle RNAs from 3 to 4 wt and ColQ^{-/-} P7 and P30 mice were extracted by using Lysing Matrix D (MP Biomedicals; Illkirch, France), proteinase K treatment and RNeasy Fibrous Tissue Mini-Kit (Qiagen) according to the manufacturer's instructions. RNA quality was determined using Experion RNA StdSens Chips, samples with RQI (RNA quality indicator) <5 were not used.

Applied Biosystems Microarrays

Microarray analysis was run as three independent triplicates per time point (T1, T2 and T3) and per cell line (wt and ColQ^{-/-} cells) using Applied Biosystems Mouse Genome Survey Microarrays, containing probes representing approximately 32,000 mouse genes from the public and Celera databases. Digoxigenin-UTP labeled cRNA was generated from 500 ng of total RNA using Applied Biosystems Chemiluminescent RT-IVT labeling kit and was purified according to the manufacturer's instructions. Each microarray was prehybridized at 55°C for 1 h in hybridization buffer with blocking reagent. 10 µg of labeled cRNA was then hybridized to each array in a 500 µl volume at 55°C for 16 h. After hybridization, the arrays were washed with hybridization wash buffer and chemiluminescence rinse buffer. Chemiluminescence detection, image acquisition and analysis were performed using Applied Biosystems Chemiluminescence Detection Kit and Applied Biosystems 1700 Chemiluminescent Microarray Analyzer following the manufacturer's protocol. Single-color chemiluminescent signals were quantified, corrected for background and spatially normalized. Applied Biosystems Expression System software was used to extract assay signal, and assay signal to noise ratio values from the micro-array images. The raw data were normalized using Lowess. The set of probes differentially expressed between wt and ColQ^{-/-} cells as well as between T1, T2 and T3 time points were determined by Student's test.

Gene Ontology

The evaluation of which gene ontology classes were differentially expressed between wt and ColQ^{-/-} samples was performed using a functional class scoring analysis as previously described (20). For each gene in a GO class, the *P* value for comparing wt *versus* ColQ^{-/-} samples was computed. The set of *P* values for a class was summarized by two summary statistics: 1) the LS summary is the average log *P* values for the genes in that class and 2) the KS summary is the Kolmogorov-Smirnov statistic

computed on the P values for the genes in that class. The statistical significance of the GO class containing n genes represented on the array was evaluated by computing the empirical distribution of these summary statistics in random samples of n genes. We considered a GO category differentially regulated if the significance level of either one of the KS or LS statistics was <0.01 (BRB ArrayTools, Class comparison). The results of this analysis are displayed as an html file (Supplemental Material).

TaqMan real-time RT-PCR

The TaqMan Low Density Array (TLDA, Applied Biosystems) was used in a two-step RT-PCR process (Marionneau et al., 2005). First-strand cDNA was synthesized from 220 ng of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). PCR reactions were then performed on TLDA with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The 384 wells of each card were preloaded with pre-designed fluorogenic TaqMan probes and primers. Probes were labeled with the fluorescent reporter 6-carboxyfluorescein (FAM[®], Applied Biosystems) at the 5'-end and with non-fluorescent quencher on the 3'-end. Three genes were selected for normalization (*HPRT1*, *GAPDH* and *SEC63*). Data were collected with Applied Biosystems SDS 2.1 software, and then analyzed with the threshold cycle (Ct) relative-quantification method (Livak and Schmittgen, 2001). Since *GAPDH* is the most uniformly distributed gene between the different conditions, we selected it for data normalization. The relative expression of each gene versus *GAPDH* ($2^{-\Delta Ct}$, ΔCt indicating normalized data: $\Delta Ct = \text{target gene Ct} - \text{GAPDH Ct}$) was calculated and then averaged for each condition.

Statistical Analysis

Data are expressed as means \pm SEM. Statistical analyses were done by pair-wise comparisons between two conditions using Student's unpaired t tests for $n \geq 30$ and Mann-Whitney U test for $n < 30$ ($P < 0.05$ considered significant).

RESULTS

1. Transcriptional profiling of ColQ-deficient cells *in vitro*

To define the network of genes under the control of ColQ during muscle cell differentiation, we aimed to compare the transcriptome of wt and ColQ-deficient muscle cell lines at three time points of myotube differentiation, called T1, T2 and T3. Cell lines and stages of differentiation have both been previously described (17, 18). Briefly, T1 corresponds to early fused myotubes, T2 corresponds to myotubes expressing AChR clusters and T3 corresponds to myotubes undergoing spontaneous contractions as well as expressing AChE clusters in wt cells but not in ColQ^{-/-} cells. The transition T1 to T2 can be correlated to the beginning of muscle innervation occurring between embryonic days E13.5 and E14 in mice. Contrariwise, the transition T2 to T3 corresponding to further maturation of muscle cells in the absence of nerve has no equivalent during mice development. mRNA were prepared from three independent wt or ColQ^{-/-} muscle cell culture aliquots and from triplicates for each time point, and then hybridized on mouse pangenomic microarrays (Applied Biosystems). After normalization of the microarray data and median filter application, the 17.035 remaining genes over 32.000 genes were classified using model-based clustering in the context of detecting differentially expressed genes between wt and ColQ^{-/-} cells or between T1 and T3. Thereby, nine clusters of genes were obtained (Figure 1A), revealing that 28% of the genes are upregulated in the absence of ColQ, 10% of the genes are downregulated when ColQ is absent and thus 62% of the genes are equally expressed between wt and ColQ^{-/-} cells (Figure 1B). Interestingly, some clusters represent genes that are differentially regulated at the three time points of muscle cell differentiation whereas other clusters correspond to genes that are differentially expressed early or late during differentiation in the absence of ColQ. These data suggest that ColQ regulates various categories of genes including genes involved in specific stages but also genes that are expressed at all stages of muscle differentiation.

2. Putative functions perturbed in the absence of ColQ *in vitro*

To determine if specific functions could be attributed to the nine clusters obtained from microarray analysis, genes within each cluster were classified depending of their Gene Ontology (GO) identity. Add cluster 1 first. No significant functions were drawn from clusters 2 and 6. Genes of olfactory

receptor activity (GO: 4984) and sensory perception (GO: 7600) classes were well represented in cluster 1 without any particular muscle function. Clusters 3, 4 and 5 contained genes that were upregulated at T1 only and represented the proliferation and cell motility functions with genes implicated in the cell cycle (GO: 7049), DNA replication (GO: 6260), cytoskeleton (GO: 5856) and microtubule organization (GO 226). The upregulation of these genes at an early phase in the absence of ColQ suggests that wt and ColQ^{-/-} myoblasts could proliferate and/or fuse differently. However, no obvious variations were observed in the timing of cell division or fusion between the two cell lines (data not shown). Cluster 7 represented a group of genes downregulated at all time points coding mostly for extracellular proteins (GO: 5576) and in particular for extracellular matrix proteins (GO: 31012). The most important function represented in cluster 8 was linked to muscle contraction (GO: 6936) and more precisely to myofibril (GO: 30016), contractile fiber (GO: 43292) and sarcomere (GO: 30017). Since this cluster 8 corresponds to genes that were upregulated during muscle cell differentiation but that were not differentially expressed between wt and ColQ^{-/-} cells, proteins implicated in contraction and muscle organization are likely not affected by ColQ deficiency. This result is in agreement with spontaneous contractions observed both in wt and ColQ^{-/-} cells and occurring at the same time in both cell lines, *ie* at T3. Finally, cluster 9 in which genes are upregulated at T2 and T3 corresponded to mitochondrion genes (GO: 5739) with well represented ion transport genes (GO: 6811) and ATPase activity genes (GO: 16887). Together, these results show that the expression of two different classes of genes is perturbed in the absence of ColQ. First, ECM genes are downregulated in ColQ-deficient cells compared to wt cells and second, mitochondrion genes are upregulated tardily in the absence of ColQ. Genes that were differentially expressed between wt and ColQ^{-/-} cells were also classified according to ingenuity functions. This classification revealed an evolution among the top functions. At T1, *cellular growth* or *skeletal and muscular system development and function* were top functions. Then, between T1 and T2, and between T2 and T3, *skeletal and muscular disorders* and *cell death* went progressively up in the top functions (Figure 2). These data suggest that ColQ deficiency affects muscle development first and is then responsible for muscle disorders during further muscle differentiation.

3. ColQ and the extracellular matrix *in vitro*

Two reasons prompted us to focus our study on genes from cluster 7. First, downregulated genes could provide insights into defects linked to the absence of ColQ. Second, it could reveal ECM genes co-regulated with ColQ. The two main classes of extracellular matrix proteins identified in this cluster were (1) components of the ECM architecture, (2) ECM enzymes and modulators. The first category was essentially composed of laminins, collagens, proteoglycans and glycoproteins. The second class included metalloproteases, growth factors and other proteins (Table 1). To validate the results obtained by microarrays, a subset of 95 various genes from microarrays including ECM genes was also quantified by Taqman Low Density Array (TLDA). In this experiment, data were normalized to Sec63 mRNAs since this specific mRNA was similarly expressed whatever the conditions according to the microarray data. For more than 91% of the genes analyzed in microarrays and TLDA, the same variations in gene expression and magnitude of the changes were observed between wt and ColQ^{-/-} cells. Together, these data highlight the reliability of microarray and TLDA techniques and show that ColQ deficiency is responsible for perturbation of gene expression of both structural and regulator components of the ECM.

Given that ColQ seems to participate to the regulation of ECM gene expression, we explored whether the absence of ColQ also perturbed dystroglycan expression, one of the main ECM receptor (21) and an indirect partner of ColQ (12). Although normal differentiation of muscle cells is characterized by an increase in DG levels *in vivo* (22) and in our wt muscle cell line, this upregulation does not occur in the absence of ColQ (Figure 3Ab). In addition, since the functional form of DG is glycosylated to allow the organization of the ECM (21), DG glycosylated forms were quantified by western blot in wt and ColQ^{-/-} cells. ColQ deficiency was found to induce a 20% decrease of glycosylated DG level at T2 and T3 compared to control condition (Figure 3B). Finally, as DG is expressed all along the muscle fibers but is preferentially accumulated at the NMJ with AChRs, we also analyzed the localization of active form of DG in myotubes forming AChR clusters. Immunofluorescence experiments performed at T2 revealed that DG co-clustered with AChRs in wt cells whereas in ColQ^{-/-}

-/- cells some AChRs were devoid of glycosylated DG (Figure 3C). Together, these results suggest that ColQ might regulate DG functional expression and localization, thus potentially controlling the molecular cascades linking ECM to cytoskeleton.

Importantly, to build the ECM, DG binds perlecan as well as laminin and agrin when normally glycosylated (22). Our mRNA analysis showed that laminin- β 1 was present in the microarray cluster 7 and was downregulated during muscle cell differentiation (Table 1). Perlecan, which bind both DG and ColQ, was significantly downregulated in the absence of ColQ at T2, with a 55% decrease (Figure 3Aa). Collectively, our data suggest that the absence of ColQ could lead to disorganization of the ECM and to a defect in its linkage to the muscle membrane.

4. ECM gene expression in ColQ-deficient mice

We thus wondered whether the modifications of ECM gene expression detected in ColQ $-/-$ cells were also observed in muscle from ColQ $-/-$ mice. Thereby, a disorganization of the ECM *in vivo* could lead to signs of dystrophy in these mice, in addition to the myasthenic syndrome. To address this issue, mRNAs from P7 and P30 gastrocnemius muscle of wt and ColQ $-/-$ mice were analyzed by TLDA for previously selected ECM genes. However, no significant decreases were observed in the absence of ColQ for the genes tested (Supplemental data1), except for the four modulators of ECM: BMP1, Mmp9, Wnt4 and myostatin (Table 2). BMP1 was decreased by 66% in P7 muscles deficient for ColQ whereas Mmp9 and Wnt4 were decreased in P30 muscles by more than 50% in the absence of ColQ. Myostatin was decreased by 45% and 60% in P7 and P30 ColQ $-/-$ muscles respectively. Some genes coding for ECM architecture proteins or ECM regulators were upregulated in the absence of ColQ. Thereby, laminin α 4, collagen 4 α 1, glypican-1, BDNF and LTBP4 mRNA levels were increased in ColQ-deficient P7 muscles compared to wt muscles. In addition, collagen 4 α 2, perlecan, biglycan and TGF β 1 were upregulated in P30 muscles in the absence of ColQ whereas agrin was upregulated both in ColQ-deficient P7 and P30 muscles.

5. Analysis of the muscle phenotype in the absence of ColQ

Animal phenotype

Genetic defects affecting ECM structure or ECM gene expression are the causes of many cases of muscular dystrophies. More, previous *in vivo* analysis showed some intrusions of Schwann cells in the synaptic cleft (13) suggesting that a number of ColQ-deficient NMJ could be denervated. Such a process could induce muscle atrophy. We therefore search for dystrophic and atrophic signs that could occur in muscles of the ColQ-deficient mice. First, dystrophic/atrophic patients generally have decreased body weight and muscle mass compared to normal patients due to muscle defects. At birth, no difference was observed in body weight between wt and ColQ^{-/-} mice (13). At However, after birth, ColQ^{-/-} mice grew slowly compared to wt littermates. At P7, the body weight of ColQ^{-/-} mice was 30% lower than wt mice and 50% lower at P30, consistent with what has been previously observed (13) (P7: wt, 4.06g ± 0.13; ColQ^{-/-}, 2.70g ± 0.25; P30: wt, 16.92g ± 1.01; ColQ^{-/-}, 8.86g ± 1.12; n=4 mice per group; Figure 4A).

Muscle phenotype

Atrophy develops with age in patients with dystrophy, fast-muscles are generally more affected than slow-muscles whereas in patients with atrophy the reverse is observed. We thus focused our analysis on sternomastoid fast-muscle (STM) and soleus slow-muscle (Sol) at P30. Analysis of muscles revealed significant decreases of 26% and 23% of the average muscle diameter in ColQ^{-/-} STM and Sol muscles, respectively (STM: wt, 1539µm ± 25.13; ColQ^{-/-}, 1138µm ± 62.52; Sol: wt, 700µm ± 19.5; ColQ^{-/-}, 538µm ± 47.4; n=3 to 4 muscles per group; Figure 4B). P30 STM muscles from ColQ^{-/-} mice had also less fibers than wt muscles (-30%, $p < 0.05$; n=3 to 4 muscles per group; Figure 4C). In addition to being less numerous, the fibers had smaller diameters both in ColQ^{-/-} STM and Sol muscles (STM: wt, 30.06µm ± 0.16; ColQ^{-/-}, 21.23µm ± 0.15; Sol: wt, 29.74µm ± 0.11; ColQ^{-/-}, 18.17µm ± 0.07; n=3 to 4 muscles per group; Figure 4D). As showed by the repartition of fiber diameters in wt and ColQ^{-/-} P30 muscles, an increase in fiber diameter variability was observed in Sol

but not in STM ColQ^{-/-} muscles although both muscles presented a reduction in fiber diameters (Figure 4E). Together, these data indicate a decrease in body weight, muscle and fiber diameters in the absence of ColQ and suggest a general atrophy in ColQ^{-/-} mice.

Fiber phenotype

To investigate the potentially dystrophic phenotype of ColQ^{-/-} muscles, more specific dystrophic histological features were explored. A widely used index of fiber type distribution is the distribution of MHC isoforms. Thereby, fast fibers expressing MHC-2B generally shift toward the expression of slower MHC isoforms (MHC-1) in dystrophic muscles. We thus analyzed the percentage of slow fibers in wt and ColQ^{-/-} STM muscle cross-sections. No difference was observed in the number of slow fiber type from STM in the absence of ColQ, whereas Sol muscles showed a 51% decrease (Figure 5A). Moreover, dystrophic muscles often have a high concentration of degenerating fibers. We studied fibers for which membrane became porous and thus are permeable to IgG from the bloodstream, an indicator for muscle fiber that are becoming necrotic. In P30 STM muscles, the percentage of necrotic fibers rose from $0.49\% \pm 0.08$ in wt to $1.67\% \pm 0.41$ in ColQ^{-/-} whereas in P7 Sol muscles, the percentage of necrotic fibers rose from $1.12\% \pm 0.08$ in wt to $2.68\% \pm 0.65$ in ColQ^{-/-} (*STM*: +239%, $p < 0.05$; *Sol*: +128%, $p < 0.05$; $n = 3$ to 4 muscles per group; $N \geq 3000$ muscle fibers; Figure 5B). Similarly, dystrophic muscles generally present high levels of regenerating fibers characterized by central nuclei. We thereby analyzed the number of fibers with central nuclei in P30 STM and Sol muscles from wt and ColQ^{-/-} mice. In P30 STM muscles, the percentage of centrally nucleated fibers rose from $3.47\% \pm 0.44$ in wt to $5.22\% \pm 0.57$ in ColQ^{-/-}, whereas in P7 Sol muscles, this percentage rose from $4.28\% \pm 1.51$ in wt to $7.16\% \pm 1.87$ in ColQ^{-/-} (*STM*: +50%, $p < 0.05$; *Sol*: +67%, *non-significant*; $n = 3$ to 4 muscles per condition, $N \geq 3000$ muscle fibers; Figure 5C). Even if there were increases in necrotic fibers and centrally nucleated fibers in the absence of ColQ, both these fibers were rather poorly represented in wt as well as in ColQ^{-/-} mice considering the total number of fibers. Lastly, no signs of fibrosis were observed at P30. Together, these data

reveal that STM and Sol do not seem to be affected by strong dystrophic signs but Sol seems to be atrophic.

To further explore whether ColQ deficiency induces some signs of dystrophy in mice, we analyzed diaphragm from ColQ^{-/-} mice as this muscle is generally the most affected by dystrophic disorders at older age. P90 diaphragm muscles from wt and ColQ^{-/-} mice were analyzed in the same way as the previous muscles. Thereby, we first determined the percentage of slow fibers in the P90 diaphragms. This fiber type was significantly decreased in ColQ^{-/-} muscles compared to wt muscle (wt, 11% ± 1.1; ColQ^{-/-} 7.2% ± 1.0; Figure 6A). We then analyzed necrotic fibers in diaphragms from wt and ColQ-deficient mice but no significant variation were observed between the percentage of necrotic fibers in wt and ColQ^{-/-} muscles (wt, 0.22% ± 0.14; ColQ^{-/-}, 0.18% ± 0.03; Figure 6A). The percentage of centrally nucleated fibers was next determined in wt and ColQ-deficient P90 diaphragm without revealing any difference between the two groups of mice (wt, 1.03% ± 0.22; ColQ^{-/-}, 1.22% ± 0.24; Figure 6A). Therefore, a switch from slow to fast fibers was observed and no signs of degeneration/regeneration were present in diaphragms from ColQ-deficient mice. Since previous analyses have demonstrated a decrease of MHC-1 concomitant to an increase in MHC-2 in atrophy (23), a more detailed analysis of MHC forms is in progress (Table3). As the progressive inability of dystrophic muscles to properly regenerate is responsible for appearance of fibrosis and fat between fibers, we finally analyzed fiber morphology using lectin-FITC which stains muscle fiber periphery as well as connective tissue. Except for the decreased diameter of the diaphragm muscle fibers (wt, 30.8 ± 0.2; ColQ^{-/-}, 28.9 ± 0.2; Figure 6B), no other changes of the muscle morphology were observed in the absence of ColQ (Figure 6C). Collectively, our data show that ColQ^{-/-} mice do not present a dystrophic phenotype but rather exhibit signs of atrophy. Whether this abnormal phenotype is specific to the diaphragm or is related to age remain to be clarified.

6. ColQ deficiency and synaptic gene expression

Besides genes mentioned above, synaptic genes are well represented in differentially expressed genes between wt and ColQ^{-/-} muscle cells and mice muscles. Interestingly, most obvious variations were upregulation of genes observed *in vitro* and *in vivo* mostly in P30 muscles (Table 4 and 5). The most striking observation concerned the genes involved in the MuSK axis: AChE, AChR subunits, MuSK and LRP4. All these genes were upregulated *in vitro* and *in vivo* except for AChR β 1 subunit at T3 and ϵ subunit at P7. Because myogenic transcription factors and more specifically myogenin are known to stimulate AChR and MuSK expression, the levels of their mRNA were analyzed. Myogenin expression was upregulated at the three time points *in vitro* and at both ages *in vivo* although this increase was only significant *in vitro* (table 6).

DISCUSSION

Lessons from the mRNA analysis

Here, we present the first study using microarrays and high output technique for gene profiling in an animal model of myasthenic syndrome and in a myogenic cell line deficient for ColQ. Two techniques, microarrays and TLDA were used to analyze the transcriptome of wt and ColQ-deficient muscles and cells in culture. Therefore an accurate comparison of the results based on these methods was possible. These techniques give similar results in terms of modifications and amplitudes of the changes in mRNA levels demonstrating the reliability of both methods. 95 mRNAs were selected for TLDA analysis *in vitro* and *in vivo*. The rationale for selecting these specific mRNAs was their relevance to the NMJ. The quantification by TLDA of 95 specific mRNAs expressed *in vitro* and *in vivo* reveals that the absence of ColQ induces rather modest differences in gene expression. Interestingly, mRNA levels were frequently oppositely regulated *in vitro* compared to *in vivo*. Most of the 95 genes analyzed *in vitro* were downregulated whereas *in vivo*, most of the same genes were upregulated. More, when regulation of mRNA levels was observed *in vitro*, changes were observed for the three time points analyzed. In contrast, *in vivo*, a percentage of changes were observed for only one of the two ages tested. All together, it suggests (1) that compensatory mechanisms occur *in vivo* that could be dictated by the nerve or/and the Schwann cell that are absent in our muscle cells in culture (2) that a dynamic process controls mRNA levels *in vivo*. mRNAs can be classified in three categories, those which are not differentially expressed, those which are upregulated and those which are downregulated in the absence of ColQ. Levels of mRNAs which are not modified are those coding for proteins involved in the structure of the muscle and its contractile properties. This seems to fit with the absence of defects in contractile properties of muscle-deficient for ColQ. *In vitro*, clusters downregulated mostly correspond to ECM and ECM receptors coding genes. Most of the mRNAs affected by the absence of ColQ are associated to functions relevant for the muscle or the

NMJ or to muscle disorders associated with atrophy, dystrophy, myopathy or stress (mitochondrial metabolism).

Molecular profiling and phenotypic analysis of ColQ-deficient muscle

The expression of a number of genes coding for ECM, ECM modulator and ECM receptor proteins are modified *in vitro* and *in vivo* although in opposite directions. It is worth noticing that ECM genes which are regulated *in vivo* are genes mostly expressed at the synapse, and therefore represent a signature of the pathology at the NMJ. These results would suggest that dystrophic signs such as necrosis, a switch from fast to slow fiber-types, fibrosis, regeneration events, inflammation and increased mitochondrial metabolism could be detected. Some of these signs were indeed observed in muscles but were more or less pronounced according to the age and the identity of the muscle. In fact, muscles did not exhibit any signs of dystrophy but presented several molecular or phenotypic hallmarks of a mild atrophy. Atrophy is one of the features associated with a general syndrome called sarcopenia that is characterized by a loss of muscle mass and strength. This feature is associated with aging, muscle disuse, neurodegenerative diseases such as spinal muscular atrophy (SMA), amyotrophic lateral sclerosis (ALS) or denervation. In the ColQ-deficient mice, the frequently observed intrusion of Schwann cell in the synaptic space could be associated to either a modification of the ECM or an adaptation to an excessive muscle stimulation by the abnormal level of ACh. Whatever the mechanisms, the abnormal structure of the NMJ would mimic a denervation process. Our results support this hypothesis and explain the presence of atrophic signs. In this context, the beneficial effect of ephedrine on myasthenic patients with CMS-1c becomes more understandable (3, 24, 25). Ephedrine is a β 2-adrenergic receptor agonist that has been shown to promote muscle growth and strength. The pathway used by this compound is still questionable but would stimulate the IGF-1/TGF- β system. We propose that ephedrine counteracts the myopathy/atrophy associated to ColQ deficiency and strengthens the synapse connectivity. In this line, it is interesting that

ephedrine treatment is also efficient in DOK-7 associated CMS, a pathology that is also characterized by myopathic defects (26). ColQ and Dok-7 CMS share a common signaling pathway relying on MuSK. An intriguing question would be to explore the role of MuSK and the signaling cascade associated in the control of muscle mass. On the other hand, muscle atrophy has been associated to motoneuron loss in neurodegenerative diseases. This process could also be an issue in CMS-1c. In our *in vivo* study and at P7, the drastic upregulation of BDNF, a retrograde factor for motoneuron that potentiates neurotransmission might be meaningful in this context and suggest that the functional connectivity between the motoneurone and the muscle is affected.

A set of synaptic mRNAs is upregulated *in vitro* and *in vivo*

One of the most striking effects revealed by this molecular study is the co-regulation of mRNAs accumulated at the muscle postsynaptic domain. These synaptic mRNAs are upregulated *in vitro* and whatever the age *in vivo*. All these mRNAs encode proteins that are expressed in the agrin/MuSK pathway. How could these mRNAs be co-regulated? The pathways that control synaptic mRNA expression involve neural secreted factors such as neuregulins and neural agrin (27). However these factors are not expressed *in vitro* in cell cultures except for a muscle form of agrin which is not significantly affected by the absence of ColQ. Transcriptional activation of synaptic genes is mediated by an N-box, a cis- element that binds transcriptional activators of the Ets family and that is present in all synaptic genes (28). However utrophin that also contains this element was not differentially expressed in the absence of ColQ raising the possibility that these genes are either not regulated by a transcriptional mechanism involving the N-box or some of these genes for unknown reason escape this common regulatory process. It has been shown that a number of synaptic genes are upregulated in denervated muscle and in some respect ColQ-deficient muscle share some phenotypic characteristics with denervated muscle. In denervated muscle, an increase in the levels of synaptic mRNAs is observed in extrasynaptic domains of the muscle and results from the

transcriptional activation of the corresponding genes by myogenin (30). This factor is a member of the myogenic transcription factors that binds the E-box, a cis-element found in the promotor of most synaptic genes (31). Its expression is stimulated in response to the inactivation of histone deacetylase 9 (HDAC9) (31). Myogenin binds E-box in cooperation with MEF2c and the expression of MEF2c is stimulated by myogenin (29). Therefore myogenic factors can regulate the expression of synaptic genes. In our study and *in vitro*, myogenin and MEF2c are both upregulated consistent with the previously described pathway. However, *in vivo* at P7, the expression of myogenin and MEF2c was not significantly changed and MEF2c but not myogenin mRNA levels were significantly downregulated at P30. In conclusion, none of the myogenic mRNAs were upregulated both *in vitro* and *in vivo* which suggests that increased levels of synaptic mRNAs may not result from the activation of a myogenic pathway. Alternatively, the increased levels of synaptic mRNAs in the absence of ColQ could result from a mechanism of mRNA stabilization. In this hypothesis, it remains to explore the RNA-binding proteins that are regulated by ColQ. Considering that ColQ is a ligand of MuSK, an attractive hypothesis would be that ColQ regulates the expression of synaptic mRNAs through this receptor. Recent data from our group supports this hypothesis (17). Two factors, 14-3-3 and erbin have been identified to bind MuSK and downregulate the levels of synaptic mRNAs (30-32). Whether one of these factors is involved in the control of synaptic mRNA regulated by ColQ remains to be explored.

Comparative analysis with AChE-deficient mice and other myasthenic syndromes

Very recently, a comparative study of the transcriptome was performed on wt and AChE knock-out mice using affymetrix chips. The values of more than 28 000 transcripts were analyzed. Interestingly,

the top functions of the most enriched clusters are related to fatty acid and lipid metabolism and to muscle contraction (33). This is not the case in our study in the absence of ColQ. AChE absence induces more stringent effect on mRNA levels compared to ColQ deficiency. Among the genes analyzed, only Syndecan-4, an ECM receptor was significantly differentially expressed in both mutant mice. More, PRiMA and ColQ mRNA levels were not significantly different in wt and AChE-deficient mice whereas AChE mRNA levels were highly upregulated in the absence of ColQ and PRiMA was downregulated at P30. Among the molecular partners of perlecan, sparse data have been obtained in a mouse model deficient in muscle perlecan. The study focus on the molecular events that underlie the muscle hypertrophy observed in these mutant mice. The authors show that fast muscles present an hypertrophy and a decreased expression of myostatin (34). Myostatin controls negatively the muscle mass and is expressed at higher levels in fast fibers(35) and loss of myostatin induces a switch from slow to fast phenotype. In ColQ-deficient mice, myostatin is downregulated *in vivo* at P7 but not significantly at P30 and upregulated *in vitro* at T1. Since myostatin is a negative regulator of muscle mass, it seems in contradiction with the loss of muscle mass (due to reduced number of fibers and the diameters of these fibers) observed in the ColQ-deficient mice.

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FIGURE TITLES AND LEGENDS

Figure 1. Gene expression profile in wt and ColQ^{-/-} muscle cells at T1, T2 and T3. (A). Gene tree for the 32000 genes analyzed in the mouse microarray. The 6579 genes differentially expressed between wt and ColQ^{-/-} (-/-) cells were classified in 8 clusters depending of their profile of expression (cluster 1-7 and 9). Cluster 8 represented genes that were similarly regulated between wt and ColQ^{-/-} cells and upregulated during muscle cell differentiation. **(B).** Classification of genes revealed that 62% of the genes were similarly regulated between wt and ColQ^{-/-} cells whereas 28% of the genes were upregulated and 10% downregulated in the absence of ColQ.

Figure 2. Ingenuity top functions at T2 and T3 *in vitro*.

Figure 3. Expression and activation of dystroglycan is controlled by ColQ. (A) Quantification of mRNAs in wt and ColQ^{-/-} cells at T1, T2 and T3 by real-time RT-PCR. Levels of mRNAs are represented as relative expression ($2^{-\Delta Ct}$ versus reference gene x 100). (a) A significant decrease of perlecan mRNAs was observed only at T2. (b) Unlike in wt myotubes, DG mRNA levels relative to Sec63 mRNAs was not upregulated during ColQ^{-/-} muscle cell differentiation. **(B)** Western blot (up panel) and quantification (bottom panel) of α -DG glycosylated form on wt and ColQ^{-/-} cells during muscle cell differentiation. GAPDH was used as a loading control. A 20% significant decrease of glycosylated α -DG was observed at T2 in the absence of ColQ. **(C)** AChR and DG clusters labeled respectively with Alexa-594 α -BTX and VIA4 antibody on T2 myotubes from wt and ColQ^{-/-} cells. α -DG and AChR clusters co-localization was less important in ColQ^{-/-} cells than in wt cells. Error bars show means \pm SEM from four to five independent experiments. * $P < 0.05$; *** $P < 0.001$; Mann Whitney's U test. Scale bar, 20 μ m.

Figure 4. wt and ColQ^{-/-} mice phenotype and muscle morphology. (A) Weight of wt and ColQ^{-/-} mice at P7 and P30. ColQ-deficient mice were 30% smaller than wt littermates at P7 and 50% smaller at P30 (n=4 mice per group). **(B)** Representation of cross-sectional diameters of wt and ColQ^{-/-} STM and Sol muscles from P30 mice. (n=3 to 4 muscles per group). **(C)** Representation of the number of fibers per STM or Sol muscle in P30 wt and ColQ^{-/-} mice. ColQ-deficient STM muscles had less fibers than wt STM muscles at P30 (-30%, $p < 0.05$), (n= 3 to 4 muscles per group). **(D)** Representation of the mean of fiber diameter in STM and Sol muscle of P30 wt and ColQ^{-/-} mice. ColQ-deficient fibers were smaller than wt fibers both in STM (-29%, $p < 0.001$) and Sol (-39%, $p < 0.001$) muscles at P30 (n=3 to 4 muscles per group; $N \geq 2000$ muscle fibers). **(E)** Distribution of cross-sectional diameters of wt and ColQ^{-/-} fibers in P30 STM and Sol muscles. An increase in fiber diameter variability was observed in ColQ^{-/-} Sol muscles but not in STM muscles at P30 (n=3 to 4 muscles per group; $N \geq 2000$ muscle fibers). For quantifications (B-D), muscle and fiber contours were labeled with lectin-FITC antibody.

Figure 5. Dystrophic histological features in ColQ-deficient STM muscle. (A-C) Representative cross-sections of STM (a, b) and Sol (c, d) muscles from wt (a, c) and ColQ^{-/-} (b, d) mice at P30. * $P < 0.05$; Student's *t* test; n=3 to 4 muscles per group; $N \geq 3000$ muscle fibers). **(A)** Slow fibers and nuclei were labeled respectively with MHC slow antibody followed by Alexa 594 secondary antibody and DAPI. Quantification of the percentage of slow fibers per muscle revealed no significant variation between wt and ColQ^{-/-} mice in P30 STM muscles but a significant 51% decrease in P30 Sol muscles. Scale bar in b for a and b and scale bar in d for c and d, 50 μm . **(B)** Necrotic fibers and fiber contours were labeled respectively with Alexa 594 goat anti-mouse IgG antibody and lectin-FITC antibody. Quantifications in P30 STM and Sol muscles showed twice more necrotic fibers in the absence of ColQ. Scale bar in b for a and b and scale bar in d for c and d, 50 μm . **(C)** Nuclei and fiber contours were labeled respectively with DAPI and lectin-FITC antibody. Quantification of the percentage of centrally nucleated fibers revealed increases of approximately 50% both in P30 STM and Sol muscles

deficient in ColQ. Arrows indicate fibers with central nuclei. Scale bar in b for a and b and scale bar in d for c and d, 25 μ m.

Figure 6. Dystrophic histological features in ColQ-deficient P90 diaphragm. (A) Quantification of the percentage of slow fibers in P90 diaphragm muscles revealed a significant decrease of 35% between wt and ColQ^{-/-} mice. Quantification of the percentage of necrotic fibers in P90 diaphragm showed no significant variation in the absence of ColQ. Quantification of the percentage of centrally nucleated fibers revealed no difference in P90 diaphragm muscles that were deficient for ColQ. **(B)** Distribution of cross-sectional diameters of wt and ColQ^{-/-} fibers in P90 diaphragms. A 35% decrease in fiber diameter was observed in the absence of ColQ but no variation in fiber diameter variability was detected ($p < 0.05$, $n \geq 2000$ fibers per group). For quantifications (A-B), slow fibers were labeled with MHC slow antibody, necrotic fibers were detected using goat anti-mouse antibody and nuclei were stained with DAPI. Muscle and fiber contours were labeled with lectin-FITC antibody. **(C)** Nuclei and fiber contours were labeled respectively with DAPI and lectin-FITC antibody. No visual disorder of the fiber membrane and connective tissue could be detected in ColQ^{-/-} diaphragm (b) compared to wt diaphragm (a). Scale bar in b for a and b, 50 μ m.

TABLES

Table 1. ECM genes downregulated in the absence of ColQ *in vitro* (Cluster 7)

Gene (Symbol)	Accession Number	Fold Change and Significance		
		RefSeq	T1	T2
1. ECM architecture components				
- Laminin				
Laminin β 1 (Lamb1) [#]	NM_008482.1	-1.40 **	-2.21 ***	-1.98 ***
- Collagens				
Collagen 2 α 1 (Col2a1) [#]	NM_031163.1	-3.32 **	-1.58 *	-1.75 *
Collagen 4 α 2 (Col4a2)	NM_009932.2	-1.39 *	-1.42 **	-1.68 *
Collagen 5 α 2 (Col5a2) [#]	NM_007737.1	-2.41 ***	-1.73 **	-1.53 **
Collagen 6 α 3 (Col6a3)	NM_001243008.1	-11.4 ***	-2.18 ***	-1.47 ***
Collagen 7 α 1 (Col7a1) [#]	NM_007738.1	-1.61 *	-2.35 *	-4.13 ***
Collagen 8 α 1 (Col8a1)	NM_007739.1			
- Proteoglycans				
Glypican 4 (Gpc4)	NM_008150.1	-3.65 ***	-1.78 *	-1.14 *
Versican (Vcan)	NM_001081249.1	-3.77 ***	-2.52 ***	-2.48 ***
Decorin (Dcn) [#]	NM_007833.1	-60.4 ***	-31.0 ***	-23.3 ***
Asporin (Aspn)	NM_025711.2	-7.94 ***	-1.74 **	-1.79 **
Proline/arginine-rich end leucine-rich repeat protein (Prelp) [#]	NM_054077.2	-16.2 ***	-7.10 **	-3.95 **
- Glycoproteins				
Nidogen 2 (Nid2) [#]	NM_008695.1	-1.48 **	-1.75 **	-1.90 ***
Microfibrillar-associated protein 2 (Mfap2)	NM_008546.2	-28.1 ***	-14.7 ***	-13.2 ***
Extracellular matrix protein 1 (Ecm1)	NM_007899.1	-3.36 **	-2.19 *	-1.5 *
Fibuline 5 (Fbln5)	NM_011812.2	-5.36 *	-2.76 *	-2.00 *

Sarcoglycan δ (Sgcd) [#]	NM_011891.2	-1.67 **	-2.28 **	-1.43 *
- Other				
Fraser syndrome 1 homolog (Fras1)	NM_175473.2	-2.60 **	-3.50 *	-1.16 *
2. ECM enzymes and modulators				
- Metalloproteases				
Matrix metalloproteinase 16 (Mmp16)	NM_019724.2	-7.74 **	-5.39 *	-2.86 *
Matrix metalloproteinase 19 (Mmp19)	NM_021412.1	-3.50 *	-1.19 *	-1.60 *
A disintegrin-like and metalloprotease with thrombospondin type1 motif, 2 (Adamts2)	NM_175643.1	-8.97 **	-2.34 *	-1.96 *
A disintegrin-like and metalloprotease with thrombospondin type1 motif, 5 (Adamts5)	NM_011782.1	-6.46 ***	-3.52 ***	-1.20 *
- Growth Factors and associated proteins				
Bone morphogenetic protein 4 (Bmp4) [#]	NM_007554.1	-23.7 ***	-4.29 ***	-3.20 ***
Fibroblast growth factor 7 (Fgf7) [#]	NM_008008.3	-5.19 ***	-7.96 ***	-6.36 ***
Brain-derived neurotrophic factor (Bdnf) [#]	NM_007540.3	-3.05 ***	-4.36 ***	-4.75 ***
Latent transforming factor beta binding protein 1 (Ltbp1)	NM_206958.1	-1.14 *	-1.58 *	-1.79 **
Latent transforming factor beta binding protein 3 (Ltbp3)	NM_008520.1	-1.04 *	-1.27 *	-1.22 *
Latent transforming factor beta binding protein 4 (Ltbp4)	NM_175641.1	-6.19 *	-4.98 *	-6.10 *
Other				
Exostoses 1(Ext1)	NM_010162.1	-1.83 ***	-1.44 *	-1.12 *
Calsyntenin 1 (Clstn1)	NM_023051.1	-1.72 **	-1.41 **	-1.34 ***

[#] gene expression variation confirmed by TLDA. Significance: *($P < 0.05$); **($P < 0.01$); ***($P < 0.001$).

Table 2. Fold changes of ECM genes and Atrogenes in ColQ-deficient muscle *in vivo*

Gene (Symbol)	Accession Number	Fold Change and Significance	
		RefSeq	P7
1. ECM architecture components			
- Collagens			
Collagen 4 α 1 (Col4a1)	NM_009931.1	+1.54 *	+1.73
Collagen 4 α 2 (Col4a2)	NM_009932.2	+1.11	+1.68 *
- Proteoglycans			
Agrin (Agrn)	NM_021604.2	+1.35 *	+1.96 *
Biglycan (Bgn)	NM_007542.4	+1.04	+1.85 *
Glypican 1 (Gpc1)	NM_016696.1	+1.35 **	-1.40
- Glycoproteins			
Nidogen 1 (Nid1)	NM_010917.2	+1.35 **	+1.50 *
Fibronectin 1(Fn1)	NM_010233.1	+1.50 *	+1.04
2. ECM enzymes and modulators			
- Metalloproteases			
Matrix metalloproteinase 9 (Mmp9)	NM_013599.2	-1.08	-2.28 *
- Growth Factors and associated proteins			
Bone morphogenetic protein 1 (Bmp1)	NM_009755.2	-3.00 *	-1.38
Brain-derived neurotrophic factor (Bdnf)	NM_007540.3	+3.30 *	-1.04
Latent transforming factor beta binding protein 4 (Ltbp4)	NM_175641.1	+1.70 *	+1.31
Transforming growth factor beta 1 (Tgfb1)	NM_011577.1	+1.19	+2.70 *
Wingless-type MMTV integration site family, member 4 (Wnt4)	NM_009523.1	-1.16	-2.07 *
Like-glycosyltransferase (Large)	NM_010687.1	+1.53 *	+1.33
3. Receptors of the ECM components			
Integrin α 7 (Itga7)	NM_008398.1	+1.10	+1.19 *

Syndecan 4 (Sdc4)	NM_011521.2	+1.54 **	+1.30
4. Atrogenes			
Myostatin (Mstn)	NM_010834.2	-1.81 **	-2.51 **

Significance: *($P < 0.05$); **($P < 0.01$); ***($P < 0.001$).

Table 3. Myosin Heavy Chain gene expression *in vivo*

Gene (Symbol)	Accession	Fold Change and Significance	
	Number	P7	P30
	RefSeq		
MHC 2A (Myh2)	NM_001039545.2	-6.44 ***	-1.31 *
MHC 2B (Myh4)	NM_010855.2	-1.26 ***	-1.12 *
MHC 2X (Myh1)	NM_030679.1	+1.01	+2.99 *
MHC 1 (Myh7)	NM_080728.2	-2.03 ***	-5.65 *

Significance: *($P < 0.05$); ***($P < 0.001$).

Table 4. Fold changes of synaptic genes in ColQ-deficient muscle cells *in vitro*

Gene (Symbol)	Accession Number	Fold Change and Significance			
		RefSeq	T1	T2	T3
1. Membrane receptors					
Acetylcholine receptor α 1 subunit (Chrna1)	NM_007389.2		+2.42 ***	+1.57 **	+1.02
Acetylcholine receptor β 1 subunit (Chrn1)	NM_009601.3		+1.59 ***	+1.25 *	-1.25 *
Acetylcholine receptor δ subunit (Chrnd)	NM_021600.2		+2.67 ***	+1.34 *	+1.23
Acetylcholine receptor γ subunit (Chrng)	NM_009604.2		+3.28 ***	+2.08 ***	+1.52 ***
Acetylcholine receptor ϵ subunit (Chrne)	NM_009603.1		+6.04 *	+2.06 **	+2.61 ***
Low density lipoprotein receptor-related protein 4 (Lrp4)	NM_172668.2		+1.38 *	+1.19	+1.22 *
Muscle specific kinase (MuSK)	NM_010944.1		+2.45 ***	+1.39 *	+1.03
Proline rich membrane anchor 1 (Prima1)	NM_133364.2		+1.08	+1.11	-6.71 **
2. ECM components					
Acetylcholinesterase (AChE)	NM_009599.3		+2.04	-1.24	+2.17 ***
Collagen 4 α 1 (Col4a1)	NM_009931.1		-1.39	-1.30	-1.13
Collagen 4 α 2 (Col4a2)	NM_009932.2		-1.39	-1.42 **	-1.68 *
Laminin α 4 (Lama4)	NM_010681.1		+1.17	+1.50 *	+1.28
Laminin β 2 (Lamb2)	NM_008483.2		+1.18 *	+1.30 **	1.06
Nidogen 2 (Nid2)	NM_008695.1		-1.69 ***	-1.69 ***	-1.83 ***
Perlecan (Hspg2)	NM_008305		+1.02	-2.29 *	-1.31
3. Receptors of the ECM components					
Dystroglycan (Dag1)	NM_010017.3		-1.03	-1.13 *	-1.38 ***
Integrin α 7 (Itga7)	NM_008398.1		+1.69 **	+1.57 ***	+1.44 **
Integrin β 1 (Itgb1)	NM_010578.1		-1.11	-1.07	+1.01
Sarcoglycan α (Sgca)	NM_009161.1		-1.07	-1.03	-1.21
Sarcoglycan β (Sgcb)	NM_011891.2		+1.15	-1.07	-1.06

Sarcoglycan δ (Sgcd)	NM_011891.4	-1.64 **	-2.28 **	-1.43 *
Sarcoglycan ϵ (Sgce)	NM_011360.2	-1.27	-1.16	+1.05
Sarcoglycan γ (Sgcg)	NM_011892.3	+2.36 ***	+1.31 **	1.31 **
Sarcospan (Sspn)	NM_010656.2	+2.00	+1.37 *	-1.07
4. Intracellular components				
Dystrobrevin α (Dtna)	NM_207650.1	+1.21	-1.31	-1.2 *
Dystrobrevin β (Dtnb)	AK085675.1	-1.31	+1.31	+1.27
Dystrophin (Dmd)	NM_007868.1	-1.04	-1.59 *	+1.47
Rapsyn (Rapsn)	NM_009023.1	+2.09 ***	+1.55 *	+1.27 *
Syntrophin α 1 (Snta1)	NM_009228.1	+1.88	+1.77	+1.81 *
Tumorous imaginal discs 1 (Tid1)	NM_023646.4	+1.20 **	+1.79 *	+1.40
Utrophin (Utrn)	NM_011682.3	+1.20	-1.01	+1.17
5. Other				
Cluster of differentiation 24 (Cd24)	NM_009846.1	-1.52 ***	+1.38 *	+1.31 **

gene expression variation confirmed by TLDA. £ gene expression analyzed by SyBR Green RT-PCR. Significance: *($P < 0.05$); **($P < 0.01$); ***($P < 0.001$).

Table 5. Fold changes of synaptic genes in ColQ-deficient muscle *in vivo*

Gene (Symbol)	Accession Number	Fold Change and Significance	
		RefSeq	P7
1. Membrane receptors			
Acetylcholine receptor α 1 subunit (Chrna1)	NM_007389.2	+1.43 *	+1.54 **
Acetylcholine receptor β 1 subunit (Chrn1)	NM_009601.3	+1.19 *	+1.17
Acetylcholine receptor δ subunit (Chrnd)	NM_021600.2	+2.24 *	+3.81 **
Acetylcholine receptor γ subunit (Chrng)	NM_009604.2	+1.04	+2.66**
Acetylcholine receptor ϵ subunit (Chrne)	NM_009603.1	-1.19	+1.85 *
Low density lipoprotein receptor-related protein 4 (Lrp4)	NM_172668.2	+1.00	+1.52 **
Muscle specific kinase (MuSK)	NM_010944.1	+1.04	+1.37 *
Proline rich membrane anchor 1 (Prima1)	NM_133364.2	+1.34	-1.55 *
2. ECM components			
Acetylcholinesterase (AChE)	NM_009599.3	+2.03 ***	+1.93 *
Laminin α 4 (Lama4)	NM_010681.1	+1.40 *	+1.10
Laminin α 5 (Lama5)	NM_001081171.2	+1.04	+1.41
Laminin β 2 (Lamb2)	NM_008483.2	+1.16	-1.07
Nidogen 2 (Nid2)	NM_008695.1	-1.02	+1.93 *
Perlecan (Hspg2)	NM_008305	+1.43	+2.04 *
3. Receptors of the ECM components			
Dystroglycan (Dag1)	NM_010017.3	+1.07	+1.12
Integrin α 7 (Itga7)	NM_008398.1	+1.10	+1.19 *
Integrin β 1 (Itgb1)	NM_010578.1	-1.03	+1.22
Sarcoglycan α (Sgca)	NM_009161.1	+1.15	-1.05
Sarcoglycan δ (Sgcd)	NM_011891.2	+1.46	-1.08
Sarcoglycan ϵ (Sgce)	NM_011360.2	+1.42	+1.19

Sarcoglycan γ (Sgcg)	NM_011892.3	+1.26	+1.20
Sarcospan (Sspn)	NM_010656.2	+1.08	-1.09
4. Intracellular components			
Downstream of kinase 7 (Dok7)	NM_172708.3	+1.02	+1.43 *
Dystrobrevin α (Dtna)	NM_207650.1	+1.17	+1.02
Dystrobrevin β (Dtnb)	AK085675.1	+1.34	+1.61
Dystrophin (Dmd)	NM_007868.1	+1.64	+1.28
Rapsyn (Rapsn)	NM_009023.1	+1.08	-1.35 *
Syntrophin α 1 (Snta1)	NM_009228.1	+1.09	+1.01
Syntrophin β 1 (Sntb1)	NM_016667.3	-1.09	+1.50
Syntrophin β 2 (Sntb2)	NM_009229.4	+1.33 *	+1.92 **
Utrophin (Utrn)	NM_011682.3	+1.45	+1.61

Significance: *($P < 0.05$); **($P < 0.01$); ***($P < 0.001$).

Table 6. Myogenic genes and regulators of myogenic factors *in vitro* (A) and *in vivo* (B)

A.

Gene (Symbol)	Accession Number	Fold Change and Significance			
		RefSeq	T1	T2	T3
Regulators of myogenic factors					
Histone deacetylase 1 (Hdac1)	NM_008228.2		-1.19	+1.05	-1.24
Histone deacetylase 2 (Hdac2)	NM_008229.2		-1.16	-1.13	-1.16
Histone deacetylase 3 (Hdac3)	NM_010411.2		+1.36 **	+1.19	+1.25 *
Histone deacetylase 5 (Hdac5)	NM_001077696.1		+1.55	+1.09	+1.54 *
Histone deacetylase 6 (Hdac6)	NM_010413.3		+1.26	+1.12	+1.31
Histone deacetylase 7a (Hdac7a)	NM_001204275.1		-1.18	+2.92	+2.02
Histone deacetylase 10 (Hdac10)	NM_199198.2		+1.12	+1.39	-1.06
Histone deacetylase 11 (Hdac11)	NM_144919.2		-1.03	+1.21	+1.18
Myogenic factors					
Myocyte enhancer factor 2b (Mef2b)	NM_008578.2		+1.18	+1.49	-1.17
Myocyte enhancer factor 2c (Mef2c)	NM_001170537.1		+1.29 **	+1.38 **	-1.16
Myocyte enhancer factor 2d (Mef2d)	NM_133665.3		-1.22	+1.10	+1.85
Myogenic factor 5 (Myf5)	NM_008656.5		+1.42	-3.24 ***	+1.02
Myogenic factor 6 (Myf6)	NM_008657.2		-1.51	+1.03	+1.08
Myogenic differentiation 1 (Myod1)	NM_010866.2		-1.25	+1.06	-1.04
Myogenin (Myog)	NM_031189.2		+2.01 ***	+1.53 ***	+1.36 **
Paired box genes					
Paired box 1 (Pax1)	NM_008780.2		+1.39 *	+1.35	+1.03
Paired box 2 (Pax2)	NM_011037.4		-1.66	+3.33 **	+1.08
Paired box 7 (Pax7)	NM_011039.2		-18.2 *	-39.4 ***	-65.7 ***

Significance: **($P < 0.01$); ***($P < 0.001$).

B.

Gene (Symbol)	Accession Number	Fold Change and Significance	
		RefSeq	P7
Myogenic factors			
Myocyte enhancer factor 2c (Mef2c)	NM_001170537.1	+1.03	-1.46 *
Myogenic factor 5 (Myf5)	NM_008656.5	+1.17	+1.46
Myogenic factor 6 (Myf6)	NM_008657.2	+1.66 *	+1.10
Myogenic differentiation 1 (Myod1)	NM_010866.2	+1.28	-1.05
Myogenin (Myog)	NM_031189.2	+1.22	+1.26
Paired box genes			
Paired box 3 (Pax3)	NM_008781.4	+2.58	-13.36
Paired box 7 (Pax7)	NM_011039.2	+1.14	+1.19

Significance: *($P < 0.05$).

SUPPLEMENTAL DATA DESCRIPTION

Supplemental data 1. Table of non-significant fold changes of ECM genes in ColQ-deficient muscle

in vivo

Gene (Symbol)	Accession Number	Fold Change and Significance	
		RefSeq	P7
1. ECM architecture components			
- Laminin			
Laminin α 2 (Lama2)	NM_008481.1	+1.10	+1.23
Laminin α 5 (Lama5)	NM_001081171.2	+1.04	+1.41
Laminin β 1 (Lamb1)	NM_008482.1	+1.14	+1.32
Laminin β 2 (Lamb2)	NM_008483.2	+1.16	-1.07
- Collagens			
Collagen 6 α 1 (Col6a1)	NM_009933.1	+1.09	+1.16
Collagen 6 α 2 (Col6a2)	NM_146007.1	+1.11	+1.21
Collagen 6 α 3 (Col6a3)	NM_001243008.1	+1.08	+1.60
Collagen 13 α 1 (Col13a1)	NM_007731.1	+1.21	+1.15
- Proteoglycans			
Glypican 4 (Gpc4)	NM_008150.1	-1.01	+1.33
Versican (Vcan)	NM_001081249.1	+1.12	+1.95
Decorin (Dcn)	NM_007833.1	-1.45	+1.23
Proline/arginine-rich end leucine-rich repeat protein (Prelp)	NM_054077.2	+1.26	+1.19
- Glycoproteins			
Sarcoglycan α (Sgca)	NM_009161.1	+1.15	-1.05
Sarcoglycan β (Sgcb)	NM_011890.2	+1.29	-1.28
Sarcoglycan δ (Sgcd)	NM_011891.2	+1.46	-1.01

Sarcoglycan ϵ (Sgce)	NM_011360.2	+1.42	+1.19
Sarcoglycan γ (Sgcg)	NM_011892.3	+1.26	+1.20
2. ECM enzymes and modulators			
- Metalloproteases			
Matrix metalloproteinase 2 (Mmp2)	NM_008610.2	+1.05	+1.04
A disintegrin and metalloproteinase domain 12 (Adam12)	NM_007400.1	-1.05	+1.38
A disintegrin-like and metalloproteinase with thrombospondin type1 motif, 2 (Adamts2)	NM_175643.1	+1.40	+1.27
A disintegrin-like and metalloproteinase with thrombospondin type1 motif, 5 (Adamts5)	NM_011782.1	-1.13	+1.22
- Growth Factors and associated proteins			
Fibroblast growth factor 6 (Fgf6)	NM_010204.1	+1.41	+1.99
Transforming growth factor beta 2 (Tgfb2)	NM_009367.1	+1.01	+1.67
Transforming growth factor beta 3 (Tgfb3)	NM_009368.1	+1.50	+1.12
Tissue inhibitor of metalloproteinase 1 (Timp1)	NM_001044384.1	+1.65	+1.17
Other			
Wingless-type MMTV integration site family, member 1 (Wnt1)	NM_021279.3	+2.02	+3.02
Fukutin related protein (Fkrp)	NM_173430.1	+1.33	-1.18
Fukutin (Fktn)	NM_139309.4	-1.18	+1.10
Protein O-linked mannanase b1, 2-N-acetylglucosaminyltransferase 1 (Pomgnt1)	NM_026651.2	+1.01	-1.14
Protein-O-mannosyltransferase 1 (Pomt1)	NM_145145.1	-1.17	-1.11
Protein-O-mannosyltransferase 2 (Pomt2)	NM_153415.1	+1.06	+1.18
3. Receptors of the ECM components			
Dystroglycan (Dag1)	NM_010017.3	+1.07	+1.12
Fibroblast growth factor receptor 1 (Fgfr1)	NM_010206.1	+1.37	+2.12
Integrin β 1 (Itgb1)	NM_010578.1	-1.03	+1.22
Syndecan 3 (Sdc3)	NM_011520.2	+1.32	+1.52

Significance: *($P < 0.05$); **($P < 0.01$); ***($P < 0.001$).

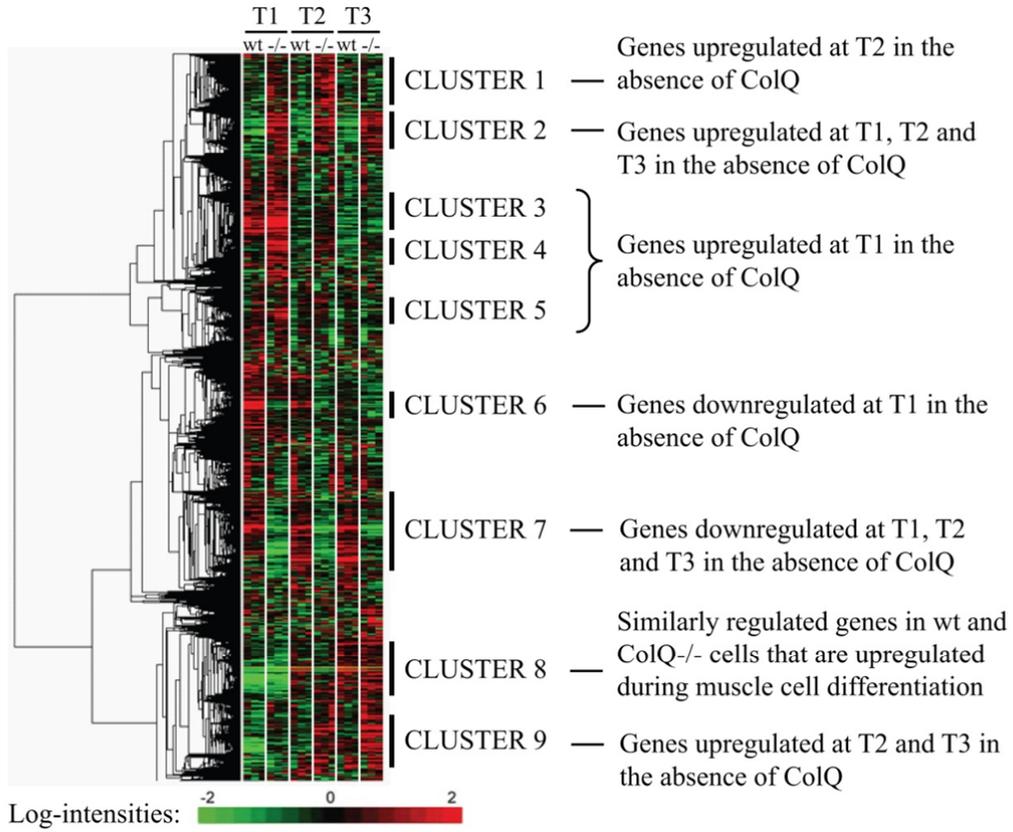
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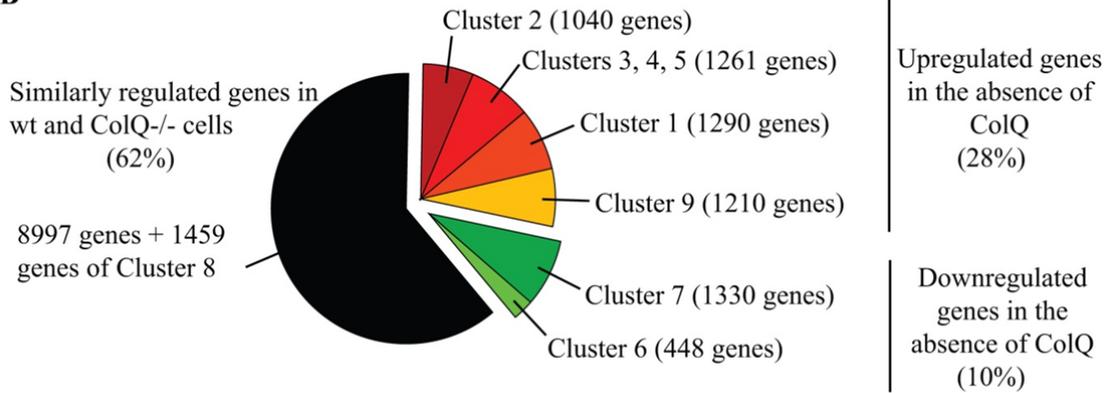
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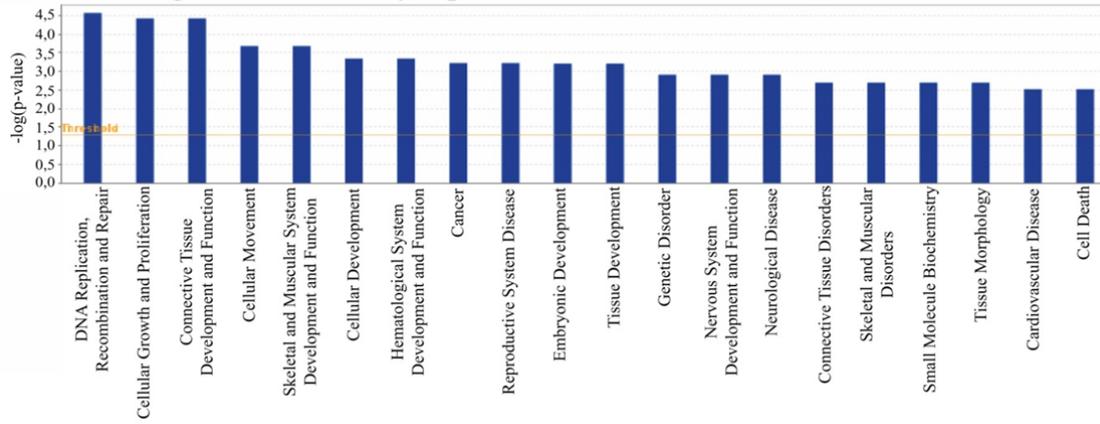
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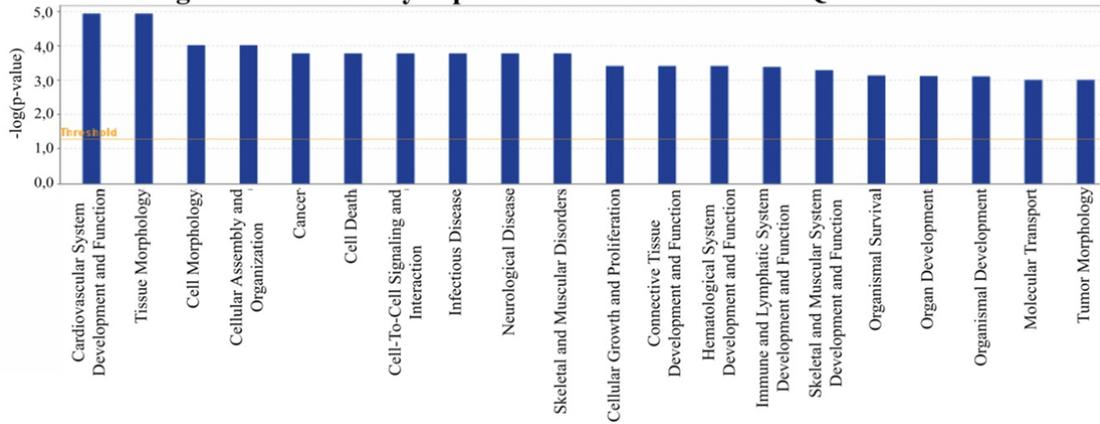
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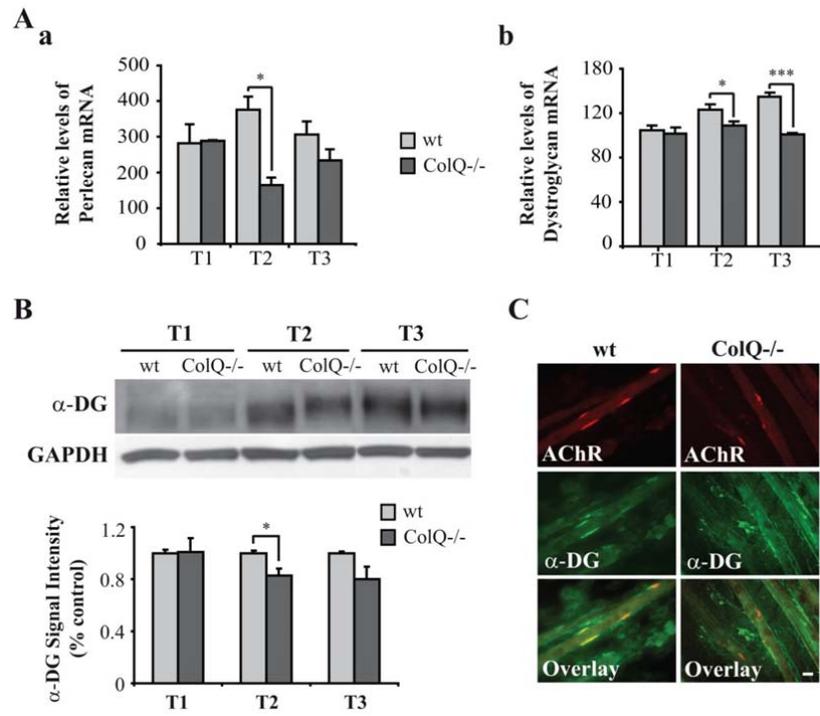


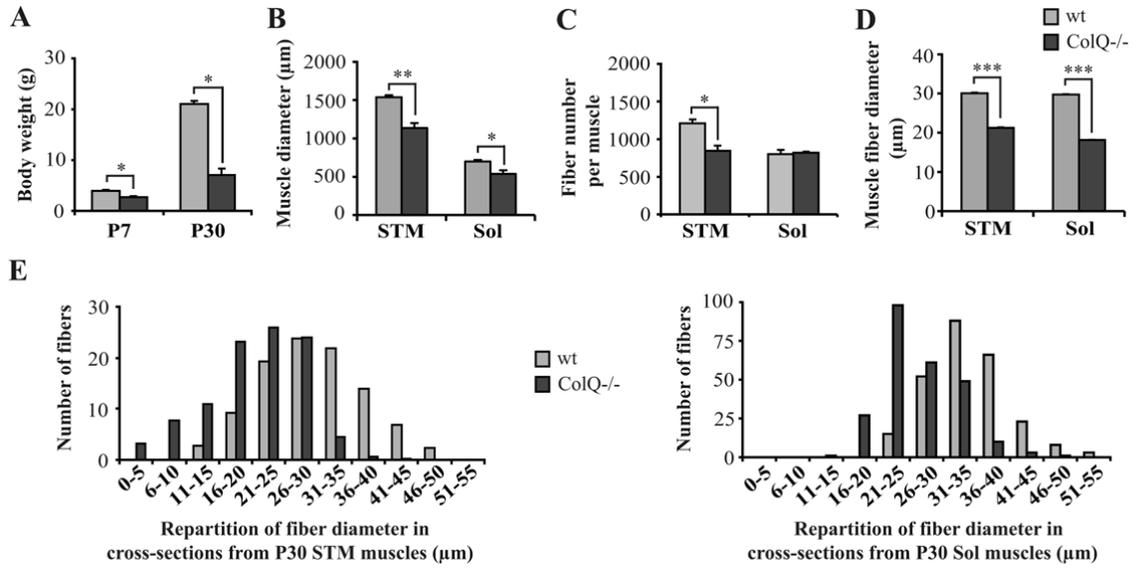
A genes differentially expressed between wt and ColQ^{-/-} cells at T2

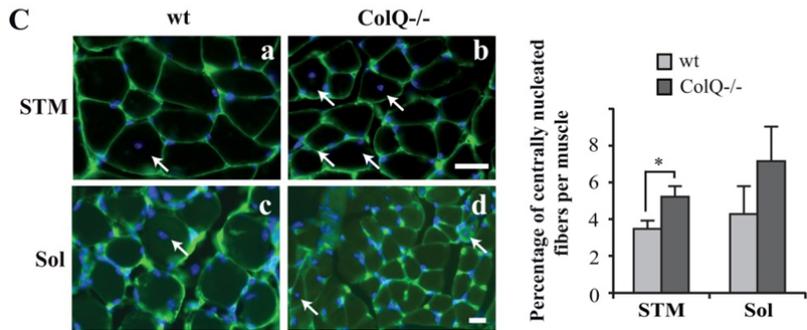
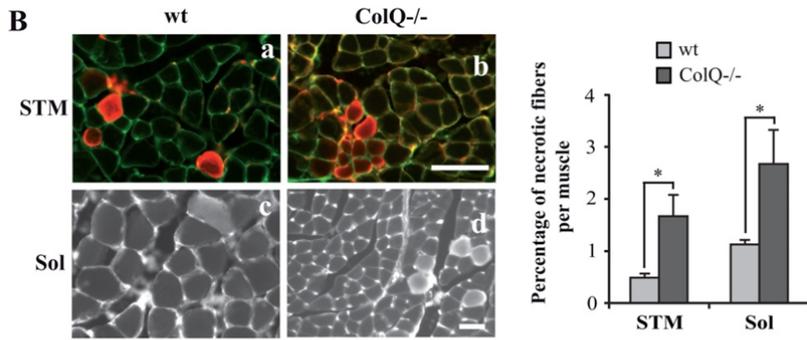
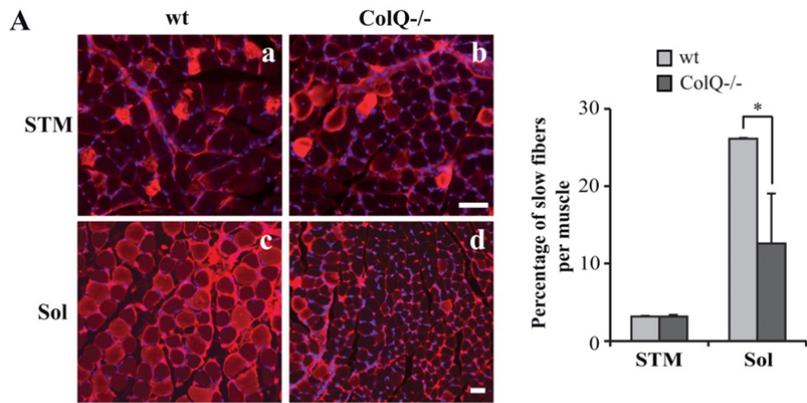


B genes differentially expressed between wt and ColQ^{-/-} cells at T3

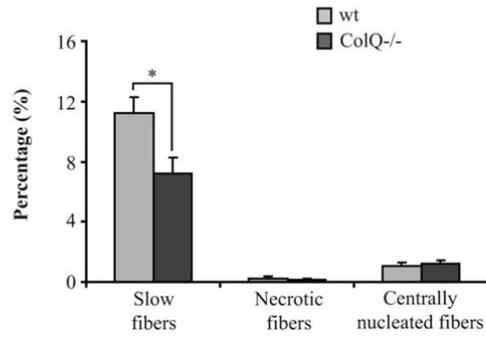




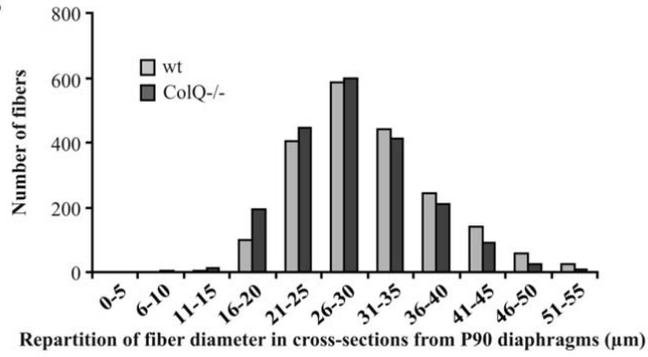




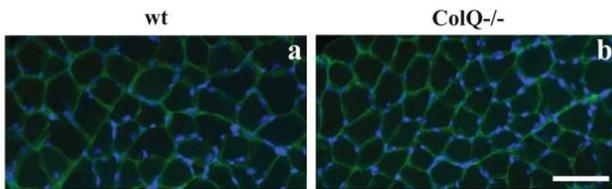
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B



C



ANNEX B: LIST OF PUBLICATIONS AND COMMUNICATIONS

Publications

Karmouch J, Delers P, Belanger G, Dobbertin A, Jasmin B, Legay C, ColQ regulates Acetylcholine receptor mRNA stability through HuR and the activation of p38: implications for Congenital Myasthenic Syndrome with Acetylcholinesterase deficiency. (Submitted to Human Molecular Genetics).

Sigoillot SM, Bourgeois F, Karmouch J, Krejci E, Chevalier C, Houlgatte R, Léger J, Legay C, Molecular and phenotypical analysis of ColQ-deficient muscles in a model of myasthenic syndrome, to be submitted to *Faseb. J* (In preparation)

Karmouch J, Dobbertin A, Sigoillot S, and Legay C, Developmental consequences of the ColQ/MuSK interactions, *Chem Biol. Interact.* Chem Biol Interact. 2013 Mar 25;203(1):287-91

Before PhD:

Zhou Y, Ma C, Karmouch J, Arabi Katbi H, Liu XJ. An Anti-Apoptotic Role for Ornithine Decarboxylase during Oocyte Maturation. *Mol. Cel. Bio.* (2009),1786-1795

Anas MK, Lee MB, Zhou C, Hammer MA, Slow S, Karmouch J, Liu XJ, Bröer S, Lever M and Baltz JM. SIT1 is a betain/proline transporter that is activated in mouse eggs after fertilization & functions until the 2-cell stage". *Development* 135, 4123-4130 (2008)

Conference communications

Karmouch Jennifer, Delers Perrine, Bélanger Guy, Dobbertin Alexandre, Jasmin Bernard, Legay Claire (2013) "Deciphering the role of ColQ in the control of Acetylcholine receptor expression", 11èmes journées annuelles de la Société Française de Myologie (Montpellier, France)

Delers Perrine, Karmouch Jennifer, Bélanger Guy, Jasmin Bernard, Legay Claire (2013) "Acetylcholine Receptor mRNA metabolism at the Neuromuscular Junction", Federation of European Neuroscience Societies (Prague, Czech Republic)

Karmouch Jennifer, Delers Perrine, Bélanger Guy, Dobbertin Alexandre, Jasmin Bernard, Legay Claire (2013) Deciphering the role of ColQ in the control of Acetylcholine receptor expression Retreat of Neuroscience group at Univ. Paris Descartes (Meudon, France)

Karmouch Jennifer, Delers Perrine, Bélanger Guy, Dobbertin Alexandre, Jasmin Bernard, Legay Claire (2013) Deciphering the role of ColQ in the control of Acetylcholine receptor expression XIV International Symposium on Cholinergic Mechanisms. (Hangzhou, China) [Prize for Best Poster]

Dobbertin Alexandre, Sigoillot M. Severine, Bourgeois Francine, Karmouch Jennifer, Legay Claire. (2012) "Role of MuSK-ColQ interactions in synaptogenesis of the neuromuscular junction", Retreat of Neuroscience group at Univ. Paris Descartes (Gif-sur-Yvette, France)

Dobbertin Alexandre, Sigoillot M. Severine, Bourgeois Francine, Karmouch Jennifer, Legay Claire (2011) “Role of MuSK-ColQ interactions in synaptogenesis of the neuromuscular junction”, Society for Neuroscience (Washington DC, USA).

Karmouch Jennifer, Sigoillot M. Severine, Dobbertin Alexandre, Bourgeois Francine, Krejci Eric, Chevalier Catherine, Houlgatte Remi, Leger Jean, and Legay Claire. (2011) “Molecular and phenotypical analysis of ColQ-deficient muscles, a model of myasthenic syndrome”, AFM Myology (Lille, France)

Before PhD:

Karmouch Jennifer (2007), “The Regulation of Protein Translation During Xenopus Oocyte Maturation”, Reproductive Biology Workshop.

Karmouch J (2006), “The role of microRNA in the regulation of protein translation during Xenopus oocyte maturation”, presented at the Ottawa Health Research Institute -Research Day

ABSTRACT

ColQ is a specific collagen that anchors acetylcholinesterase (AChE) in the synaptic cleft of the neuromuscular junction (NMJ). The importance of AChE-ColQ complex in the physiology of this synapse has been highlighted by the identification of *COLQ* mutations in the human gene, leading to a congenital myasthenic syndrome (CMS) with AChE deficiency. The lack of AChE has been incriminated for the symptoms observed in patients along with NMJ defects in the CMS mouse model (ColQ-deficient). However, symptoms observed in the patients and mouse model of CMS with AChE deficiency are complex and AChE deficiency cannot account for all of them. We have demonstrated that ColQ could play a role *per se* in synapse formation which would explain some of the defects observed in patients and model mice. Indeed, we have shown that ColQ controls the clustering of Acetylcholine Receptors (AChR) and the expression of a number of specific synaptic genes. The most striking effect of the absence of ColQ is an upregulation of all AChR subunit mRNAs correlated by an increase in their protein levels. Preliminary results indicate that AChR mRNA upregulation is not transcriptional.

This thesis deciphers the mechanisms that drive AChR mRNA upregulation in the absence of ColQ and the pathways that connect ColQ to the AChR RNA metabolism. Accordingly, we hypothesize that the absence of ColQ induces an upregulation of the stabilization of AChR subunit mRNAs, a post-transcriptional mechanism mediated by HuR. HuR is an RNA binding protein which stabilizes its target transcript by binding AU-rich elements (AREs) in their 3'UTR. HuR is critical during skeletal myogenesis and post-synaptic NMJ formation due to its stabilization of such transcripts as myogenin, MyoD and AChE.

In this study, we show for the first time that a post-transcriptional mechanism of AChR mRNA stabilization is responsible for the ColQ mediated increase of AChR mRNAs. In support of these findings, the absence of ColQ also increased HuR mRNA and protein levels. We demonstrate that HuR is capable of binding to conserved ARE elements in the 3'UTR of AChR subunit mRNA. HuR's interaction with AChR mRNA increased the stability of the transcripts, resulting in an increase in mRNA levels. Three major conclusions emerge from my thesis: we provide evidence that (1) in addition to transcriptional and assembly regulation of AChR, post-transcriptional mechanisms of AChR mRNA exist (2) ColQ regulates HuR mediated AChR stability through MuSK and (3) the p38 signalling pathway controls the levels of HuR in a ColQ dependent manner. Collectively, our data provides insight into the muscle signaling pathways which are affected by ColQ mutations leading to CMS with AChE deficiency. Thus, we have identified specific new molecular targets that may become important for the development of therapeutic interventions for patients with CMS.

Key words: ColQ; Acetylcholine receptor; mRNA stability; HuR; neuromuscular junction; congenital myasthenic syndrome with AChE deficiency

RESUME

ColQ est un collagène spécifique qui ancre l'acétylcholinestérase (AChE) dans la fente synaptique de la jonction neuromusculaire (JNM). L'importance du complexe AChE-ColQ dans la physiologie humaine de cette synapse est soulignée par l'identification de mutations dans le gène codant pour ColQ qui conduisent à un syndrome myasthénique congénital (SMC) associé à une déficience en AChE. Le déficit en AChE a, jusqu'à présent, été considéré comme l'unique facteur responsable des symptômes observés chez les patients ainsi que des défauts de la JNM chez le modèle de souris SMC (souris déficiente pour ColQ). Toutefois, ces symptômes sont complexes et l'absence d'AChE ne peut probablement pas expliquer tous les symptômes. Nous avons montré auparavant que ColQ participait à la formation de la synapse ce qui expliquerait les symptômes observés chez les patients et la souris modèle. En effet, nous avons pu montrer que ColQ contrôle l'agrégation du récepteur à l'acétylcholine (RACH) et de l'expression de gènes spécifiques de la synapse. En particulier, nous avons montré *in vitro* et *in vivo*, que l'absence de ColQ induit une augmentation du niveau des ARNm codant pour toutes les sous-unités de RACH et une expression réduite du niveau de leurs protéines. Des résultats préliminaires indiquent que cette augmentation de ces ARNm n'est pas transcriptionnelle.

L'objectif de cette thèse est d'expliquer les mécanismes qui induisent l'augmentation du niveau des ARNm de AChR en l'absence de ColQ et les voies de signalisation qui relie ColQ au métabolisme des ARN du RACH. Notre hypothèse de travail a été que l'absence de ColQ stimule la stabilisation post-transcriptionnelle des ARNm codant pour les sous-unités du RACH via la protéine HuR. HuR est une protéine qui stabilise les ARNm quand elle se fixe sur les AU-rich element (ARE) dans la séquence 3'UTR. HuR est une protéine clé dans la myogenèse et la formation de la JNM parce qu'elle stabilise de manière post-transcriptionnelle de nombreux transcrits tels que myogénine, MyoD et AChE.

Dans cette étude, nous montrons pour la première fois qu'un mécanisme post-transcriptionnel de stabilisation des ARNm est responsable de l'augmentation du niveau des ARNm du RACH via ColQ. De plus, nous constatons qu'en absence de ColQ, il y a une augmentation aux niveaux d'ARNm et de protéine de HuR. HuR est également capable de se lier au domaine ARE dans le 3'UTR des ARNm des sous-unités de AChR. De plus, l'interaction entre HuR et les ARNm du RACH augmente la stabilité et par conséquent les niveaux des transcrits du RACH.

Trois conclusions importantes ressortent de ma thèse : nous démontrons que (1) en plus de la régulation transcriptionnelle, il existe des mécanismes de régulation post-transcriptionnelle du RACH (2) ColQ régule la stabilité des ARNm RACH via HuR médiée par MuSK (3) la voie de signalisation p38 contrôle les niveaux de HuR de manière dépendante de ColQ. Ensemble, ces résultats donnent un aperçu des voies de signalisation du muscle qui sont affectées par les mutations de ColQ conduisant à des SMC avec une déficience en AChE. Nos résultats mettront en évidence des nouvelles cibles moléculaires spécifiques qui peuvent conduire au développement des interventions thérapeutiques dans le cadre de myasthénies congénitales.

Mots clés: ColQ; Récepteur à l'acétylcholine; Stabilité des ARNm; HuR; Jonction neuromusculaire; Syndrome myasthénique congénital associé à une déficience en AChE