Congenital Myasthenic Syndromes and the Therapeutic Modulation of the Neuromuscular Junction

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Author's Declaration

This thesis is submitted to Newcastle University for the degree of Doctor of Philosophy. The research was performed at the John Walton Muscular Dystrophy Research Centre between the years 2015-2019, under the supervision of Prof Hanns Lochmüller, Dr Roger Whittaker, Prof Rita Horvath and Dr Veronika Boczonadi.

I can certify that the material offered in this thesis has not been previously submitted by me for a degree or qualification in this, or any other university.

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Many people have contributed to my PhD work since I began this project in September 2015.

Firstly, I wish to express my gratitude to Prof Hanns Lochmüller. Ever since I approached Hanns in 2014 as a junior doctor with an interest in the neuromuscular junction, he has been unfailingly supportive. His encouragement gave me the confidence to apply for the ABN fellowship, and he supported me at every stage of the application process. He encouraged me to take on laboratory work, even though I had limited experience in this; an aspect which has multiplied my skill-set and hugely improved the breadth of my project. His effectiveness, foresight and belief in me and other members of his team has been inspiring. Hanns has given me so many opportunities throughout my time working with him, including attending and presenting at conferences, being named on grant applications and introducing me to experts in the field. He has driven all aspects of this project forward, and his impact not only on this work but on my career path is immeasurable.

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I also had the opportunity to work with Prof Rita Horvath throughout this project. Rita has taken this project in new directions, and allowed me to expand my knowledge of inherited neuropathies and neurogenetics. Her clinical knowledge is vast and she has been an amazing person to learn from both as a clinician and as a scientist.

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I was incredibly lucky that shortly after I started my fellowship, Prof Clarke Slater decided to come out of retirement and set up a neuromuscular junction research facility within Newcastle University. Having Clarke around has changed my approach in all aspects of my research, and I have learned so much from him, not only about the neuromuscular junction but also about conducting research with integrity and taking pride in my work. His infinite enthusiasm for the neuromuscular junction and meticulous methodology has been inspiring to witness. I have loved our discussions about the principles of neuromuscular transmission and the unanswered questions in the study of the neuromuscular junction, and I hope they will continue.

Finally, I would like to thank my husband Olan, who moved from Ireland to Newcastle so I could undertake this project. Thank you for your continuous support.

Summary

Impairment of neuromuscular transmission at the neuromuscular junction is a primary pathomechanism of many human conditions. The congenital myasthenic syndromes (CMS) are caused by primary genetic defects which reduce the efficacy of neuromuscular transmission. CMS are increasingly diverse, both phenotypically and genetically, and the study of this group of disorders has improved our understanding of the role of neuromuscular junction proteins in health and disease.

For most subtypes of CMS, symptomatic treatments are available. However, these are poorly understood and often limited by systemic side effects. In many CMS, sympathomimetics such as salbutamol and ephedrine lead to clinical benefit. However, the reason for this clinical benefit is unknown. Using animal models, this research aimed to explore the mechanisms underlying improved muscle strength from sympathomimetics in CMS. Experiments in zebrafish models revealed that salbutamol alters many aspects of neuromuscular junction development. Follow-up studies in the mouse model of end-plate acetylcholinesterase deficiency revealed that salbutamol leads to structural neuromuscular junction alterations which are primarily postsynaptic.

The identification of novel CMS genes has been accelerated by the use of next generation sequencing. Genetic sequencing of undiagnosed patient cohorts lead to the identification of a novel presynaptic CMS subtype caused by mutations in *SLC5A7*, encoding the presynaptic choline transporter. Mutations in *SLC5A7* were previously associated with an inherited motor neuropathy, and this finding expanded the overlap between disorders of the neuromuscular junction and of the motor nerve. In addition, through analysis of a patient cohort with CMS and episodic apnoea, I identified genetic, phenotypic and neurophysiological characteristics which provide mechanistic insights into this phenomenon.

Understanding the mechanisms of neuromuscular junction dysfunction and of its therapeutic modulation are essential to facilitate earlier diagnosis and the development of targeted therapies for the wide range of disorders in which the neuromuscular junction is implicated.

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Abbreviations

3,4 DAP	3,4 diaminopyridine
6mwt	6 minute walk test
AAV	Adeno associated virus
AC	Adenylyl cyclase
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChR	Acetylcholine receptor
ADL	Activities of daily living
AKAP	A kinase anchoring protein
ALS	Amyotrophic lateral sclerosis
βAR	β adrenergic receptor
cAMP	Cyclic adenosine monophosphate
ChAT	Choline acetyltransferase
ChT	Choline transporter
CK	Creatine kinase
CMAP	Compound muscle action potential
CMS	Congenital myasthenic syndrome
CMS-EA	Congenital myasthenic syndrome with episodic apnoea
CMT	Charcot Marie Tooth disease
ColQ	Collagen Q
CRD	Cysteine rich domain
CREB	cAMP response element binding protein
DGC	Dystrophin associated glycoprotein complex
Dok7	Downstream of kinase 7
Dvl	Dishevelled
ECM	Extracellular matrix
Epac	Exchange protein directly activated by cAMP
EPC	End-plate current
EPP	End-plate potential
FVC	Forced vital capacity
GARS	Glycyl tRNA synthetase

GPCR	G protein coupled receptor
hpf	Hours post fertilisation
HMSN	Hereditary motor sensory neuropathy
LABA	Long acting beta agonist
LEMS	Lambert Eaton myasthenic syndrome
LG-CMS	Limb girdle congenital myasthenic syndrome
LRP4	Low density lipoprotein receptor related protein 4
MAPK	Mitogen activated protein kinase
MG	Myasthenia gravis
MHC	Myosin heavy chain
MRC	Medical research council scale
MRI	Magnetic resonance imaging
MuSK	Muscle specific receptor tyrosine kinase
$Na_v1.4$	Voltage gated sodium channel
NMJ	Neuromuscular junction
NT	Nerve terminal
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PJ	Postjunctional folds
PKA	Protein kinase A
QMG	Quantitative myasthenia gravis score
RNS	Repetitive nerve stimulation
SABA	Short acting beta agonist
SFEMG	Single fibre electromyography
SMA	Spinal muscular atrophy
SMALED	Spinal muscular atrophy with lower extremity predominance
SMN	Survival motor neuron
SNAP25	Synaptosome associated protein 25
SNARE	Soluble NSF attachment protein receptor
SNS	Sympathetic nervous system
SR	Sarcoplasmic reticulum
TSC	Terminal Schwann cell

VAChT	Vesicular acetylcholine transporter
VGCCs	Voltage gated calcium channels

Contents

Chapter 1. Introduction

1.1 The Neuromuscular Junction and Neuromuscular Transmission

Motor nerves convey their signals to their target muscle fibres at highly specialized chemical synapses - neuromuscular junctions (NMJs). The last thirty years have seen great advances in our understanding of the cellular and molecular events taking place during development, maturation and maintenance of the NMJ *in vitro* and *in vivo* in a variety of species (Hall and Sanes, 1993; Sanes and Lichtman, 2001). Indeed, much of what we understand about synapse development and chemical transmission in neurobiology as a whole are due to the study of this relatively large and easily accessible synapse.

1.1.1 Neuromuscular Transmission

In all vertebrates, the process of neuromuscular transmission involves the release of the neurotransmitter acetylcholine (ACh) from the presynaptic nerve terminal onto the muscle fibre to trigger excitation and contraction. The NMJ is highly specialised to ensure this takes place quickly (less than a millisecond) and reliably (Figure 1) (Slater, 2008).

In the motor nerve terminal, ACh is packed into membrane bound vesicles (synaptic vesicles) or "quanta". When a motor nerve action potential arrives at the nerve terminal, it causes the opening of voltage-gated calcium channels, and an influx of Ca^{2+} ions. This increase in Ca^{2+} concentration causes synaptic vesicles to fuse with the presynaptic membrane and release ACh into the synaptic cleft, also known as "quantal release" (Del Castillo and Katz, 1954; Fatt and Katz, 1952).

The released ACh then diffuses across the synaptic cleft, and binds to the ligand gated ACh receptors (AChRs), which are highly concentrated on the surface of the muscle membrane (10,000 per μm^2) (Matthews-Bellinger and Salpeter, 1983). The binding of ACh induces a conformational change in AChRs, causing them to briefly open.

Open AChRs are approximately equally permeable to Na^+ and K^+ and the resulting flow of ions is determined by electrochemical gradients (Takeuchi and Takeuchi, 1959). However, the opening of AChRs allows much more Na^+ in than K^+ ions out; it is the net influx of Na^+ which constitutes the resulting inward current, known as the end-plate current (EPC) (Fatt

and Katz, 1951). As the bound ACh dissociates from the AChRs it is rapidly hydrolysed by acetylcholinesterase (AChE) in the synaptic cleft (Eccles et al., 1942; Gašperšič et al., 1999).

The influx of Na^+ ions leads to transient local depolarisation of the muscle membrane, known as the end-plate potential (EPP), which is typically 25-45mV (Fatt and Katz, 1951; Wood and Slater, 1995). This EPP leads to the opening of voltage-gated $Na_v1.4$ channels in the muscle fibre (Boyd and Martin, 1956). In order to generate an action potential, the EPP must reach a threshold of depolarisation, at which positive charge entering the muscle membrane is greater than that leaving the surrounding membrane (Jack et al., n.d.). In mammalian muscle, an action potential typically occurs when the membrane potential reaches -55mV (Wood and Slater, 1995). However, at the NMJ the threshold for action potential is much less, being approximately 50% less than is required in a non-junctional region (Wood and Slater, 1997). This is due to two main structural adaptations at the postsynaptic membrane; the high concentration of $Na_v1.4$ channels, and the presence of postsynaptic folds (see section 1.1.2.3).

In order to ensure that this process occurs in a reliable way, transmission at the NMJ has a high safety factor. This can be defined as the ratio of the number of quanta of ACh released to the number of quanta required to generate an action potential in the muscle fibre (Wood and Slater, 1997). Under normal conditions, more ACh is released by a motor nerve action potential than is required to initiate a muscle action potential. This is estimated to be by a factor of four, depending on the experimental conditions in which it is tested (Boyd and Martin, 1956, p.; Harris and Ribchester, 1979; Wood and Slater, 1997). During high frequency activity, the quantal content may decline significantly in a normal NMJ (Van der Kloot, 1991). The safety factor means that even under these conditions, a nerve impulse will still lead to muscle contraction. In spite of this large reserve capacity of neuromuscular transmission, there are many pathological situations in which that reserve reaches its limit and transmission is significantly impaired, resulting in fatigable muscle weakness.

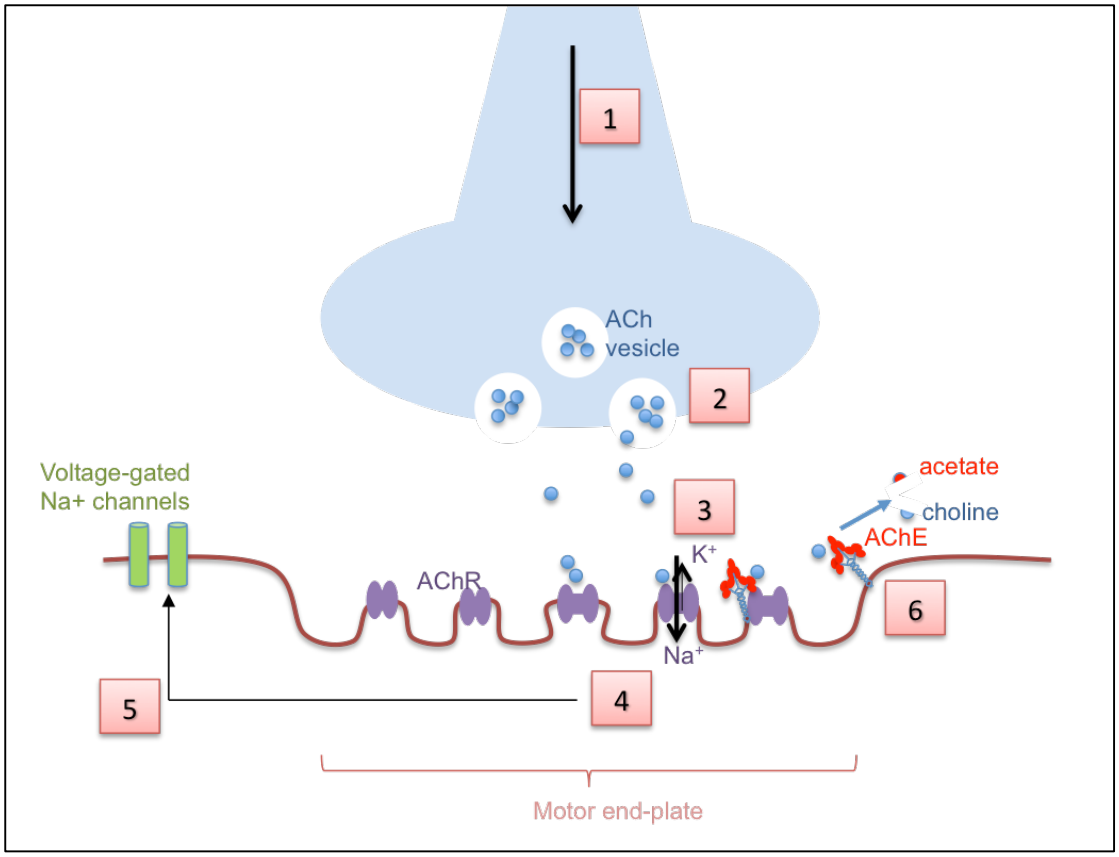

Figure 1: Synaptic transmission at the neuromuscular junction. 1. Motor neuron action potential initiates synaptic transmission in the terminal bouton. 2. Vesicles containing ACh are released by the presynaptic nerve terminal within 100µs of nerve impulse. 3. ACh binds to the transmembrane AChRs in a hydrophobic pocket formed at the interface between adjacent subunits, producing a configurational change to a more open hydrophilic channel that favours passage of ions into the muscle fibre. 4. An influx of Na ions causes a transient depolarisation of the muscle membrane (the end-plate potential) 5. The end-plate potential initiates the opening of the ion channels in the muscle fibre – the $Na_v1.4$ voltage gated channels, and initiates an action potential in the muscle fibre. 6. The action of ACh is rapidly terminated by the action of the AChE anchored on the basal lamina, which splits ACh into acetate and choline. The choline is subsequently transported into the nerve terminal where it is recycled.

1.1.2 The impact of NMJ structure on neuromuscular transmission

The NMJ is a tripartite synapse, comprised of the motor neuron, muscle fibre and Schwann cell. Each of these components has a highly specialised structure which influences the efficacy of neuromuscular transmission.

1.1.2.1 Synaptic Vesicle Exocytosis

The efficacy of exocytosis of synaptic vesicles in response to a motor nerve impulse is an important determinant of neuromuscular transmission. The process of synaptic vesicle

exocytosis is complex and involves interaction between two sets of proteins: V-SNARES, which are in the vesicular membrane, and T-SNARES, which are in the nerve terminal membrane (Sudhof, 2004). Three essential proteins of membrane fusion are the V-SNARE protein synaptobrevin/VAMP (vesicular associated membrane protein), and the T-SNARE proteins SNAP-25 and syntaxin-1 (Südhof and Rizo, 2011). Synaptic vesicles are primed for release at the active zone by partial SNARE and Sec1/Munc18-like complex assembly before calcium triggering (Südhof and Rizo, 2011). The speed and precision of subsequent exocytosis are mediated by synaptotagmin and its cofactor complexin (Südhof, 2012). Complexin binds to the primed vesicles transforming them into an active state ("superpriming") and clamps them for release. Ca^{2+} binding to synaptotagmin then triggers binding to the SNARE complex and opening of the fusion pore (Südhof, 2012; Zhou et al., 2017). After exocytosis, vesicles are re-endocytosed, recycled, and refilled with ACh (Sudhof, 2004).

1.1.2.2 The Acetylcholine Receptor

Each mature AChR is a complex of five subunits, 2α, β, ε and δ. ACh binds at two sites, the interfaces between the α and δ subunits, and between the α and ε subunits (Figure 2) (Gu and Hall, 1988). When both sites are bound by an ACh molecule, the AChR undergoes a conformational change to rapidly open the channel (Matsubara et al., 1992). There is an efflux of K^+ and an influx of Na^+ through this open channel. The channel closes again after approximately 1ms (Matsubara et al., 1992). During maturation of NMJs, the AChR subunits switch from containing a γ subunit to an ε subunit (Gu and Hall, 1988). The conductance and opening properties of the channel is affected by this molecular switch. In 2αβδγ AChRs, opening time is prolonged meaning that more Na^+ enters the muscle fibre (Mishina et al., 1986).

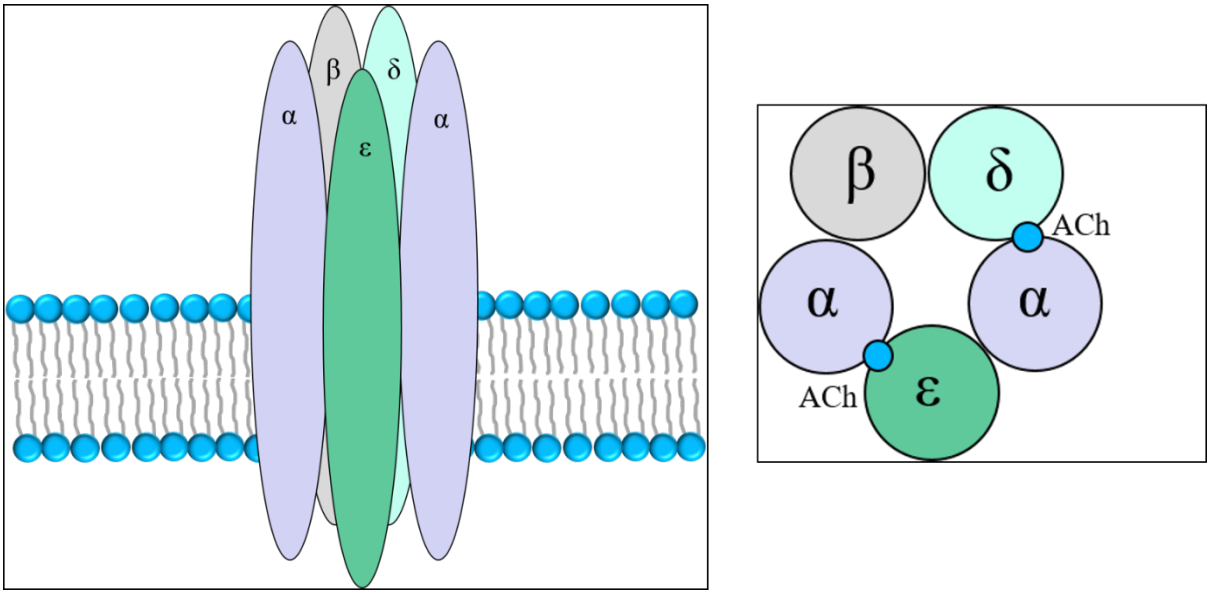

Figure 2: Schematic of the Acetylcholine Receptor in the postsynaptic membrane. A. The five subunits (2α, β, δ, ε) span the membrane and enclose the ion pore. B. View along the axis of the pore demonstrating binding sites of acetylcholine (ACh).

1.1.2.3 The Postsynaptic Folds

The postsynaptic membrane is extensively invaginated with folds extending into the cytoplasm (Figure 3). These have an important impact on the reliability of neuromuscular transmission. At the crests of the folds and extending partly down the fold walls, AChRs are highly concentrated. The depths of the folds contain a high concentration of $Na_v1.4$ channels. The high concentration of AChR at the crests of the folds means ACh binds and the EPC enters at the fold crests. As the current travels towards the cytoplasm it must therefore move down a thin fold of membrane, which has high resistance, and which amplifies the depolarisation of $Na_v1.4$ channels at the depths of the folds (Martin, 1994). As a result of the folds, the threshold at the NMJ is lowered, and fewer AChRs need to be opened in order to generate a muscle fibre action potential than would be the case in the absence of these folds (Slater, 2008).

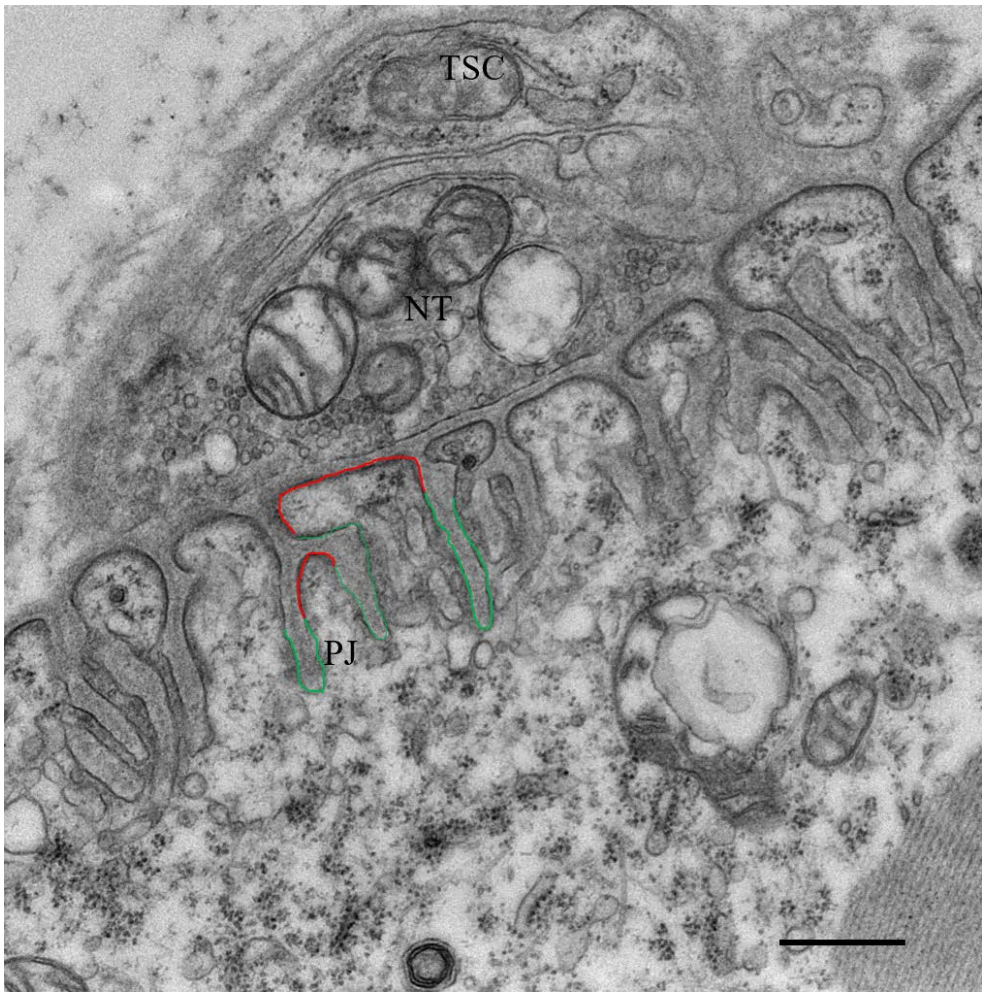

Figure 3: Electron micrograph of a mouse neuromuscular junction showing anatomy of the postsynaptic folds. **AChRs (red) are concentrated at the crests of the folds and appear electron dense, while NaV1.4 channels (green) are concentrated in the depths of the folds. NT: nerve terminal; TSC: terminal Schwann cell; PJ: postjunctional folds. Scale bar = 500nm**

1.1.2.4 Acetylcholinesterase

Asymmetric AChE at the NMJ acts to rapidly hydrolyse and terminate the action of ACh, allowing its re-uptake and recycling in the presynaptic nerve terminal (Katz and Miledi, 1973). In developing NMJs, AChE is dispersed along the muscle fibre. As synapses mature, AChE becomes highly concentrated at the NMJ where it is anchored by collagen Q (ColQ) (Krejci et al., 2006). AChE needs to be kept at high density to prevent depolarizing blockade of the post-synaptic membrane channels and neuromuscular transmission failure (Martinez-Pena y Valenzuela and Akaaboune, 2007). The mechanisms whereby AChE is localized and retained at the NMJ include binding to ColQ, perlecan and α-dystroglycan (Arikawa-Hirasawa et al., 2002; Martinez-Pena y Valenzuela and Akaaboune, 2007).

Uptake by the nerve terminal of choline resulting from hydrolysis of acetylcholine (ACh) by AChE from the synaptic cleft is undertaken by the high affinity choline transporter (ChT). Choline acetyltransferase (ChAT) catalyzes the synthesis of ACh from acetyl-CoA and choline. These newly synthesised molecules of ACh are then collected into synaptic vesicles by the vesicular ACh transporter (VAChT).

1.1.3 Signalling pathways at the Neuromuscular Junction

The assembly, maintenance, and plasticity of NMJs are differentially regulated by numerous signalling pathways activated by the cross-talk among the three partners of the NMJ (motor nerve ending, muscle fibre, and terminal Schwann cell (TSC)). These pathways play important and intersecting roles at the NMJ during development (embryonic and early postnatal life), adulthood, and aging.

1.1.3.1 Signalling pathways in mammalian neuromuscular junction assembly

The formation of the NMJ requires the orchestration of several levels of organization, not least the precise juxtaposition and contact between presynaptic nerve terminals and the postsynaptic apparatus. This orchestration occurs in a series of overlapping steps starting before birth and continuing for several weeks after it. A high density (>10,000 μm^{-2}) of AChRs at synapses is required to initiate a synaptic action potential in the muscle fibre (Slater, 2008). Conversely, in the rest of the fibre, the density of AChRs has to be kept low in order to allow complete maturation of the NMJ (Cohen and Fischbach, 1977; Hartzell and Fambrough, 1973).

NMJs do not develop at random locations in muscles; rather, they are assembled in a narrow central region of the muscle fibre, so that many NMJs are located in a row, forming an end-plate band across the fibre (Kummer et al., 2006). Even before arrival of the motor nerve, in the step known as “prepatterning”, clusters of AChRs can be seen to localize along the central region of the muscle fibres (Kummer et al., 2006). Several muscle intrinsic proteins are essential for this prepatterning process, in particular the muscle specific receptor tyrosine kinase (MuSK), the low-density lipoprotein receptor related protein-4

(LRP4) and the membrane associated protein rapsyn (Figure 4). Mutation of MuSK or LRP4 prevents prepatterning (Kim and Burden, 2008a; Weatherbee et al., 2006).

Following innervation, some of these prepatterned AChR clusters are actively incorporated into developing NMJs, supporting the view that postsynaptic muscle intrinsically defines its central region for ingrowing axons (the myocentric model) (Flanagan-Steet et al., 2005). The arriving motor nerve terminals, and the ACh released by them, subsequently stabilize the innervated AChR clusters, and cause the dispersal of non-synaptic AChR clusters.

The neuronal factor agrin, is a key player in this stabilization. Agrin binds the pre-formed MuSK-LRP4 complex to initiate a cascade of signalling pathways leading to synaptic differentiation and stabilization of the AChR clusters (Figure 4) (Zhang et al., 2008). Exogenous agrin is sufficient to induce AChR clusters and their postsynaptic specialization *in vitro* when applied to non-innervated myotubes (Bowen et al., 1996) and ectopically in adult muscle (Jones et al., 1997). Agrin deficient mice fail to form NMJs and die at birth (Gautam et al., 1996).

MuSK (muscle-specific kinase) is a transmembrane receptor kinase which is a key orchestrator in NMJ development (Figure 5). MuSK is essential for the formation of aneural AChR clusters (Lin et al., 2001). In addition, MuSK is the central component in the agrin-LRP4-MuSK receptor complex which initiates postsynaptic specialisation, clustering and anchoring AChRs in the postsynaptic membrane (Kim et al., 2008; Kim and Burden, 2008b). In cultured myotubes, activated MuSK is sufficient to form AChR clusters. MuSK can also induce the recruitment of subsynaptic nuclei and synapse-specific transcription of AChR genes (Moore et al., 2001). Agrin-activated MuSK sets up signalling loops that are not only used to control its own expression but also that of the ε and δ AChR subunit, AChE and utrophin (Jones et al., 1999; Lacazette et al., 2003). Neuregulins had been proposed to be the factor controlling expression of AChR subunit genes in fundamental myonuclei, however, postsynaptic differentiation has since been shown to occur in the absence of neuregulin receptors (Escher et al., 2005).

Low-density lipoprotein receptor-related protein 4 (LRP4) is a coreceptor for agrin and MuSK, which is necessary for the tyrosine phosphorylation of MuSK and subsequent downstream signalling. In addition, prepatterning requires LRP4 to bind MuSK (Kim et al.,

2008, p. 4; Weatherbee et al., 2006). LRP4 is expressed not only in skeletal muscle but also in motor nerves.

A further key protein which is essential for MuSK activity is Dok-7 (downstream of tyrosine kinase-7). Dok-7 is a cytoplasmic adaptor-like protein which is expressed in skeletal muscle and cardiac muscle (Okada et al., 2006). Dok-7 binds the cytoplasmic region of MuSK to induce its tyrosine phosphorylation and activation (Herbst and Burden, 2000). The formation of the agrin-LRP4-MuSK complex also causes Dok-7 phosphorylation, which creates binding sites for Crk and CrkL which may themselves play a role in synapse development (Hallock et al., 2010). Overexpression of Dok-7 using AAV vectors leads to stable NMJ enlargement and is beneficial in mouse models of Amyotrophic Lateral Sclerosis and Emery-Dreifuss muscular dystrophy (Arimura et al., 2014; Miyoshi et al., 2017).

Rapsyn (43kDa receptor associated protein of the synapse) is a cytoplasmic protein which binds tightly to AChRs to cluster them at high density (Tintignac et al., 2015). It also binds to dystroglycan, and through this action is thought to scaffold AChRs to the actin cytoskeleton (Bartoli et al., 2001).

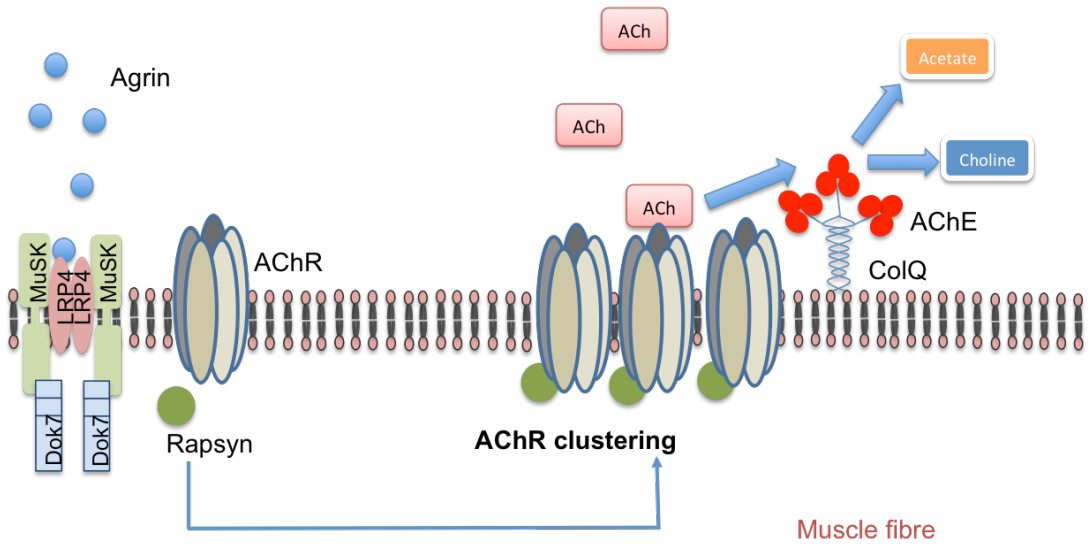

Figure 4: The agrin/LRP4/MuSK pathway regulates NMJ assembly. Neuronal agrin binds with the co-receptor LRP4 to initiate MuSK autophosphorylation and activation. Subsequently, this initiates interactions between MuSK and other key post-synaptic proteins including Dok-7, which are crucial for its catalytic activity and for downstream signalling. Several downstream signalling pathways lead to synaptic differentiation and stabilisation of the AChR clusters.

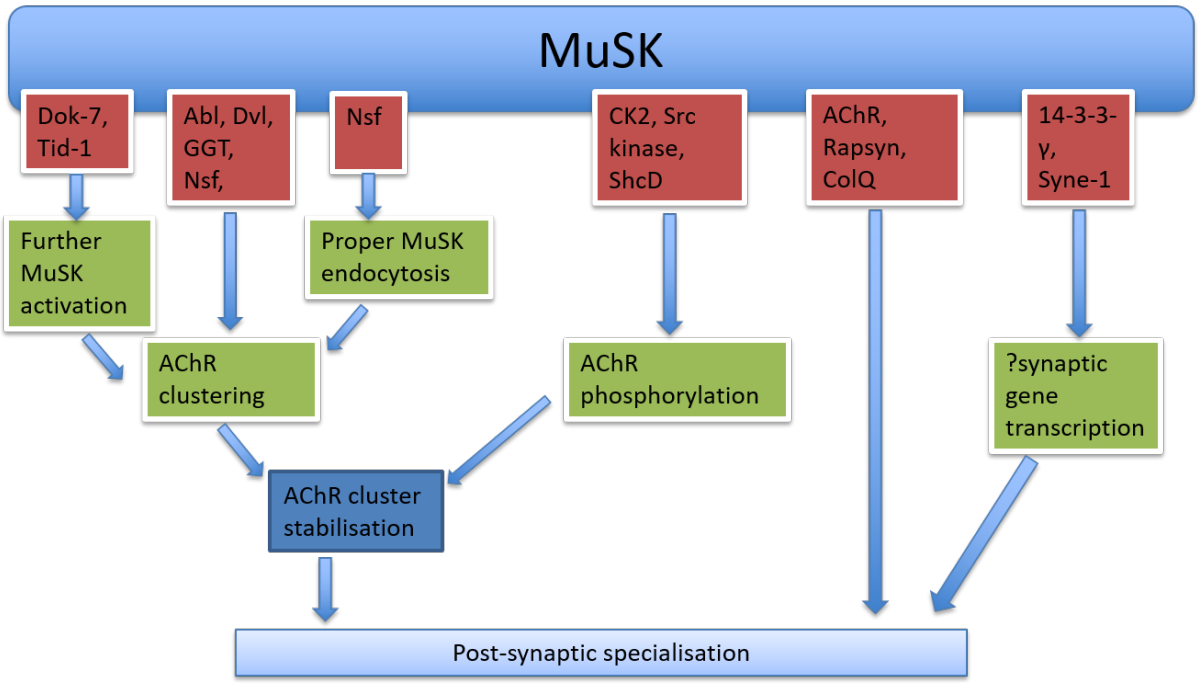

Figure 5: MuSK is a key orchestrator of NMJ development. MuSK is a receptor for neuronal agrin to initiate a plethora of downstream pathways leading to AChR clustering and stabilisation. MuSK interacts with Dok-7 and Tid-1 to initiate its own activation. In addition, MuSK interacts with scaffold proteins including rapsyn, ColQ and AChR. MuSK also interacts with proteins thought to regulate synaptic gene expression, suggesting a role in synapse-specific transcription.

In addition to these key proteins, several other signalling pathways are now known to be involved in NMJ assembly.

Growing evidence suggests the involvement of Wnt proteins in NMJ development (Figure 6). Wnt proteins are evolutionarily conserved, secreted glycoproteins expressed as 19 different isoforms in mouse and humans, which are involved in a wide range of developmental processes (Koles and Budnik, 2012). Wnt proteins activate a number of distinct signalling pathways, including the canonical Wnt pathway, the divergent canonical Wnt pathway, the planar cell polarity (PCP) pathway and the calcium Wnt signalling pathway (Cadigan and Peifer, 2009).

The first indication that Wnt signals were involved in NMJ development was the discovery of the Wnt receptor cysteine rich domain (CRD) on MuSK (Masiakowski and Yancopoulos, 1998). Subsequently, Dishevelled (Dvl), a protein activated by several Wnt signalling pathways, was found to be a binding partner of MuSK (Luo et al., 2002). Wnt4, 9a and 11

enhance AChR clustering in muscle cells *in vitro* (Barik et al., 2014; Strochlic et al., 2012). Wnt4 and Wnt11r in zebrafish, and Wnt3 in mice contribute to AChR prepatterning (Jing et al., 2009; Strochlic et al., 2012, p. 4). In addition, β-catenin, which is promoted by canonical Wnt signalling, has been shown to bind to rapsyn (Zhang et al., 2007). In mice with muscle β-catenin gain- or loss-of-function, a critical level of β-catenin expression was found to be required for proper pre and postsynaptic specialisation (Liu et al., 2012; Wu et al., 2012). Finally, mice lacking the CRD domain of MuSK lack normal prepatterning of AChRs, have outgrowth of motor axons that bypass AChR clusters, and develop myasthenic muscle weakness. This demonstrates a critical functional role of Wnt-MuSK interaction. Overall, although the detailed mechanisms are not fully understood, Wnt morphogens are emerging as key players in formation of the NMJ.

An additional auxiliary NMJ signalling pathway is via neuregulins. These are trophic factors expressed in muscle and nerves, and are important for cross talk between nerve, muscle and the TSC. Neuregulins activate the receptor tyrosine kinases ErbB2, ErbB3, and ErbB4, all of which are present at the NMJ (Tintignac et al., 2015). They have been shown to play a role in AChR expression, myogenesis and muscle repair after injury (Ford et al., 2003; Jo et al., 1995).

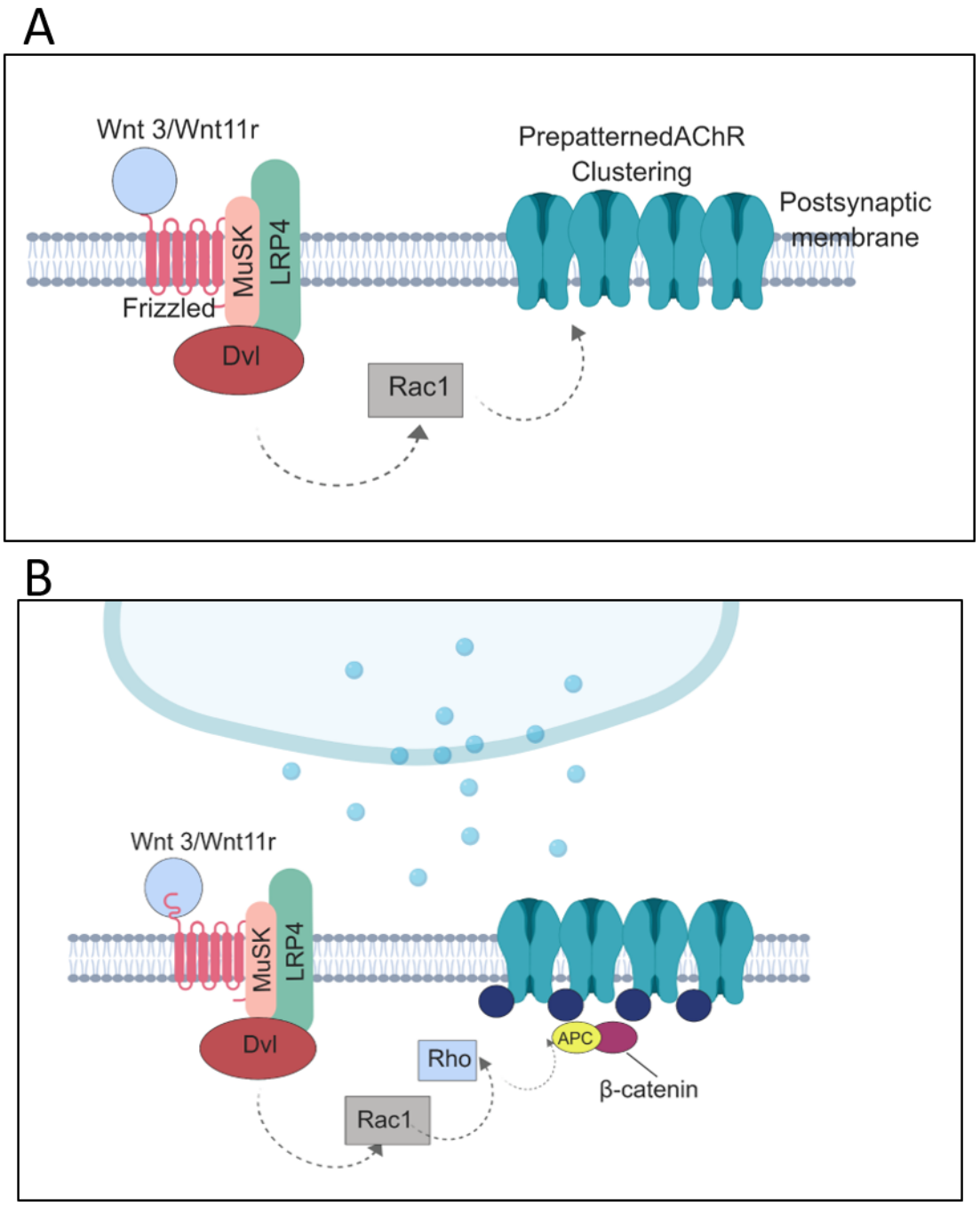

Figure 6: The role of Wnts in AChR clustering at the vertebrate neuromuscular junction. A. Wnt3 (in mice) and Wnt11r (in zebrafish) contribute to the induction of AChR (acetylcholine receptor) prepatterned clustering independent of agrin or innervation. B. Wnts also play a role in the stabilisation of AChRs in the presence of neural agrin at the time of innervation. Dvl: dishevelled. Adapted from Coles and Budnik 2012.

1.1.3.2 Signalling during NMJ maturation

NMJs undergo a long, activity-dependent maturation process, which involve structural and molecular changes at several levels. The number of presynaptic nerve terminals reduce as they compete with each other to innervate each muscle fibre. The folds in the postsynaptic

membrane develop, and the extracellular matrix (ECM) matures. In addition, the primitive plaque-like end-plate transforms into a mature pretzel shape. Finally, the TSC caps the contact between the nerve and the muscle (Sanes and Lichtman, 2001).

Synapse elimination

In the early stages of NMJ development multiple motor axons may contact a single AChR cluster. As the final steps of NMJ formation are completed (in the first 2-3 weeks after birth in rodents) this multiple innervation is reduced to a single motor axon per AChR cluster (Brown et al., 1976). Each motor nerve terminal competes locally at the end-plate, and the superseding axon is the axon with the strongest input and delivering the most efficient synaptic communication (Darabid et al., 2014). During elimination, the immature plaque-like end-plate becomes remodelled into a pretzel (Slater, 1982). At the same time, fetal 2αβδγ AChRs are replaced by adult 2αβδε AChRs (Mishina et al., 1986). A similar molecular switch occurs at this time in the Na_v1 channels, which change from the $Na_v1.5$ isoform to the $Na_v1.4$ isoform (Lupa et al., 1993). TSCs regulate and enhance this process. They destroy and phagocytose losing axons as they retract from the muscle cell surface (Bishop et al., 2004). In addition, by detecting the levels of transmitter released from each competing nerve terminal, TSCs promote the survival of the strongest input (Smith et al., 2013).

Maturation of the cytoskeleton controlling AChR stability

The synaptic basal lamina is formed from a highly differentiated network of ECM proteins which are synthesised and secreted by the muscle fibre. This structure plays a pivotal role in the maturation of the synapse. The main protein constituents of the ECM are laminin and collagen IV, but at the NMJ it also contains ColQ, perlecan, agrin, fibronectin, nidogen and others (Singhal and Martin, 2011). Laminin α and β chains play an important role in the development of both pre- and post-synaptic structures, with each laminin chain playing a specific role. In Laminin β2 knockout mice, NMJs have a reduced number of synaptic vesicles and loss of active zones (Noakes et al., 1995). In laminin α4 knockout mice nerve terminals are smaller and the active zones and postsynaptic apparatus is not properly aligned (Patton et al., 2001). Another important finding is that Laminin β2 mice lack postjunctional folds, as

do laminin α2 mice. This suggests that the distribution of each laminin chain at the NMJ may regulate the development of postsynaptic folds, which are important amplifiers of neuromuscular transmission (Rogers and Nishimune, 2017).

Collagen IV α2, α3, and α6 chains, as well as collagen XIII are concentrated at NMJs in mammals. Collagens are expressed late in NMJ development (after 3 weeks postnatally in mice) and are particularly important for presynaptic specialisation (Fox et al., 2007). ColQ is the collagenic subunit of AChE which anchors asymmetric AChE in the basal lamina (Rotundo, 2003). Mice with a knockout of ColQ lack all AChE at the NMJ, but despite this are viable and survive into adulthood (Feng et al., 1999). In addition, ColQ has been shown to bind MuSK and plays a role in postsynaptic differentiation through this interaction (Sigoillot et al., 2016, 2010). ColQ also binds to perlecan, which also plays a role in localising AChE to the NMJ (Singhal and Martin, 2011). It is not known precisely how ColQ, perlecan and MuSK interact to localise AChE to the NMJ, but it may be that they form a coordinated protein complex (Singhal and Martin, 2011).

The Dystrophin—Glycoprotein Complex

The dystrophin associated glycoprotein complex (DGC), which includes dystrophin, utrophin, sarcoglycans, sarcospan, dystrobrevin, syntrophin and three dystroglycans, forms an important part of the subsynaptic apparatus. Several studies have shown that the DGC is important for synaptic, as well as muscle, stability. Deletion of components of the DGC impairs the postsynaptic development of the NMJ. For example, misglycosylation of α-dystroglycan causes fragmented and unstable NMJs to form (Hara et al., 2011). Deletion of α-dystrobrevin reduces AChR density and destabilises AChR clusters (Grady et al., 2003). These abnormalities occurred in the context of a relatively mild muscle phenotype, suggesting they may not be secondary to disrupted myofibre architecture. More severe NMJ disassembly is found in triple knockout mice lacking α-dystrobrevin, dystrophin and utrophin, suggesting coordinated roles for these proteins in NMJ stability (Grady et al., 2000). However, it is not clear from this study whether these NMJ defects were more than would be expected given the severely dystrophic phenotype. In addition, the localisation of AChRs at the crests of the folds arises through their interaction with the DGC (Apel and

Merlie, 1995). The stabilisation of Na_v1 channels at the depths of the folds is through the interaction with β-spectrin and ankyrinG (Bewick et al., 1996; Wood and Slater, 1998).

1.1.3.3 Signalling pathways in NMJ stabilisation

The development of the NMJ can be thought of as a lifelong process. Although most mature NMJs persist throughout adulthood, their stability requires a dynamic equilibrium, with rapid remodelling in response to activity and aging. Most of the molecules involved in NMJ maintenance are those that also are essential for NMJ development as has been shown by induced suppression of agrin, MuSK and LRP4 in postnatal mice (Tintignac et al., 2015).

Metabolic stabilisation of AChRs

The high density of AChRs in the postsynaptic membrane is determined by their rates of insertion into and removal from the postsynaptic membrane (Figure 7). The stability of AChRs can be measured by repeated labelling of AChRs with α-bungarotoxin, and measurement of this labelling over time *in vivo* (Bruneau et al., 2005). This has demonstrated that AChRs in mice have a half-life of 10-14 days (Bruneau et al., 2005). The metabolic stabilisation (prolongation of the half-life) of AChRs is through their links with the actin cytoskeleton via the DGC (Tintignac et al., 2015). After they are endocytosed, AChRs can be degraded by autophagic decay or can be recycled back to the postsynaptic membrane (Bruneau et al., 2005). Several studies have demonstrated that protein kinase A (PKA) can promote AChR recycling back to the postsynaptic membrane (Martinez-Pena y Valenzuela and Akaaboune, 2007; Nelson et al., 2003; Röder et al., 2010). It is not known precisely which phosphorylation events control the decision making between AChR recycling or degradation. However it is possible that AChR itself is the target, given that it is known to be phosphorylated by PKA (Huganir and Miles, 1989; Miles et al., 1987; Nimnual et al., 1998). PKA has been shown to be anchored in close vicinity to vesicles containing recycled AChRs, where it was anchored by rapsyn (Röder et al., 2010). Agrin also promotes the recycling of AChRs after their internalisation, and through this action can metabolically stabilise AChR clusters (Brenner and Akaaboune, 2014).

Subsynaptic nuclei and the regulation of NMJ gene expression

As AChRs become innervated, genes coding for components of the NMJ become exclusively expressed in subsynaptic nuclei (Schaeffer et al., 2001). Several signalling pathways are implicated in regulating this synapse-specific gene expression. In particular, the expression of AChR subunit genes is maintained in subsynaptic nuclei despite repression in extrasynaptic nuclei through muscle activity (Sanes and Lichtman, 1999; Schaeffer et al., 2001). The targeted expression of many synaptic genes is thought to be due to the N-box, a six base pair promoter element which is shared by *CHRNE*, *CHRND*, *ACHE* and *MUSK* (Schaeffer et al., 2001).

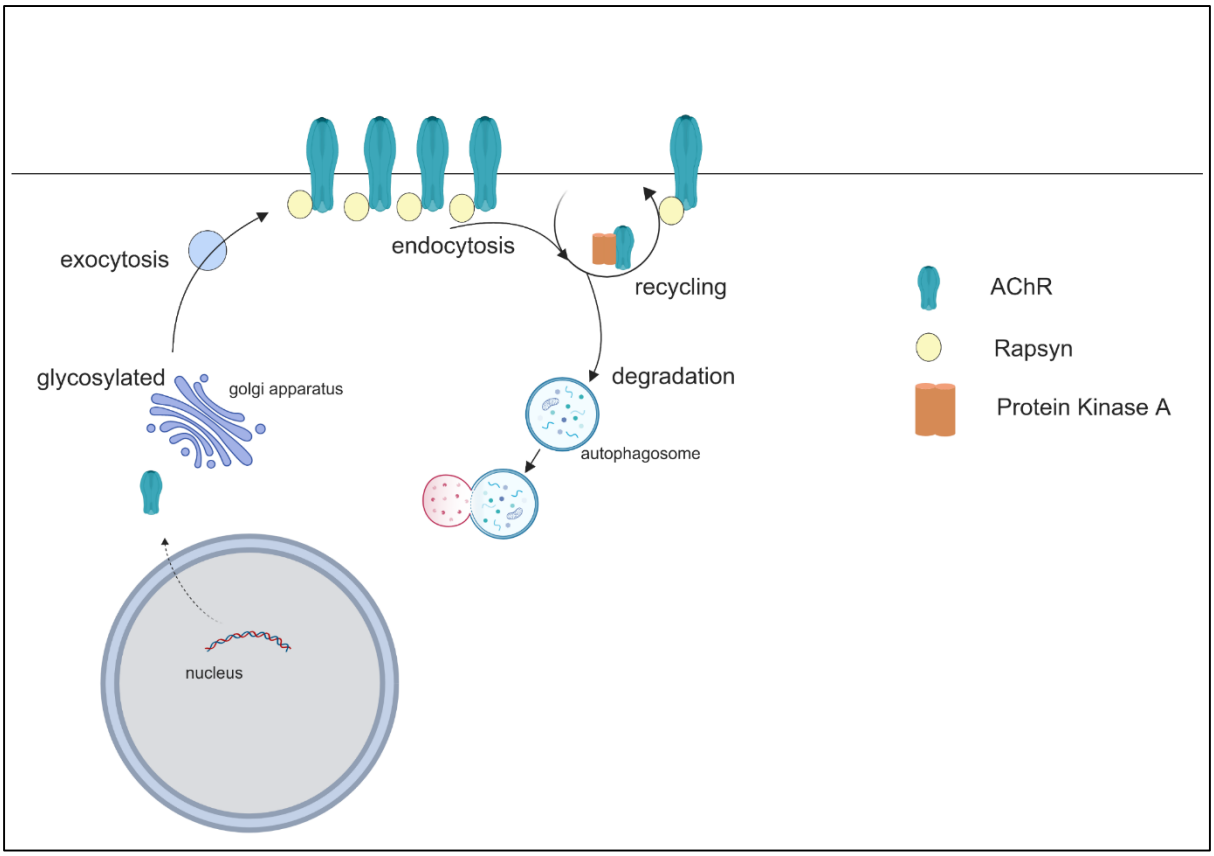

Figure 7: The life cycle of AChRs at the NMJ. AChRs are assembled in the endoplasmic reticulum and glycosylated in the golgi apparatus before being delivered to the postsynaptic membrane and clustered by the agrin/MuSK/LRP4 complex. After 10-14 days, AChRs are endocytosed and either recycled through pathways involving protein kinase A and rapsyn, or degraded by autophagy. Adapted from Rudolf et al 2014.

1.2 The NMJ in Human Disease

Many human diseases have, as either a primary or secondary component of their pathology, interruption of normal neuromuscular transmission due to defects at the NMJ. The advances in identifying the molecular pathways of neuromuscular transmission have been paralleled by the remarkable diversity of NMJ pathologies that has emerged over recent decades. The experimental accessibility of NMJs and availability of animal models has facilitated research into the mechanisms underlying selective vulnerability of distal axons and NMJs in these disorders.

1.2.1 Autoimmune diseases of the NMJ

The most common type of diseases resulting in NMJ dysfunction are acquired autoimmune disorders. Autoantibodies targeting AChRs, MuSK or rarely LRP4 lead to acquired autoimmune myasthenia gravis (MG) which presents with fluctuating skeletal muscle weakness, usually involving susceptible muscle groups, and has an estimated prevalence of 77.7 per million persons.[16,17] In AChR-MG, which accounts for 85% of cases, IgG1 and IgG3 AChR antibodies predominate (Rødgaard et al., 1987). These trigger two pathogenic mechanisms: 1) they bind directly to AChRs causing endocytosis and degradation; 2) they activate the complement pathway leading to formation of the membrane attack complex (Verschuuren et al., 2013). This results in the removal of AChR and AChR associated proteins, and in the loss of postsynaptic folds, reducing the safety factor and resulting in myasthenic weakness (Verschuuren et al., 2013). AChR-MG can be early-onset (usually before 40 years) or late onset (>60 years), and typically involves ocular muscles at onset with subsequent generalised weakness (Gilhus and Verschuuren, 2015). Some studies have indicated minor pupillary abnormalities in MG patients which may be due to the presence of skeletal ciliary muscle (Bibby et al., 1995; Dutton et al., 1982). However, visual acuity is unaffected in MG (Nair et al., 2014).

Antibodies to AChR are detected in 80-85% of cases of MG (Rodríguez Cruz et al., 2015; Vincent and Newsom-Davis, 1985). In 70% of the cases which are seronegative for AChR antibodies, antibodies to MuSK are detected (Vincent et al., 2008). Clinically MuSK-MG

differs from AChR-MG, with more involvement of neck, bulbar (often tongue atrophy) and respiratory muscles (Farrugia et al., 2006). In addition, MuSK-MG rarely occurs over the age of 60 years, having a peak age of onset in the 4th decade (Guptill et al., 2011). The lack of IgG deposits or of AChR loss at NMJs from MuSK-MG patients, lead to debate as to whether MuSK antibodies play a role in disease pathogenesis or are merely a disease marker (Selcen et al., 2004). However, several important studies demonstrated that passive transfer of MuSK IgG, and specifically IgG4, from MuSK MG patients to mice could induce myasthenic weakness and caused AChR loss (Cole et al., 2010, 2008; ter Beek et al., 2009). Subsequent studies demonstrated that MuSK IgG4 blocked the assembly of the agrin-LRP4-MuSK complex, impairing MuSK's ability to stabilise AChR clustering (Ghazanfari et al., 2018).

Autoantibodies to LRP4 are detected in approximately 10% of cases with double seronegative MG (Higuchi et al., 2011; Zhang et al., 2012). These patients share some clinical similarities with MuSK-MG, with prominent involvement of respiratory and bulbar muscles, in-keeping with the fact that LRP4 forms a complex with MuSK (Verschuuren et al., 2013). LRP4 antibodies inhibit the agrin-LRP4 interaction and agrin induced AChR clustering (Higuchi et al., 2011; Zhang et al., 2012).

Current treatment for MG includes AChE inhibitors (e.g. pyridostigmine) for symptomatic control, immunosuppressants (e.g. corticosteroids, azathioprine, methotrexate, mycophenolate, ciclosporin and rituximab) for maintenance therapy and immunomodulatory therapies (intravenous immunoglobulin or plasma exchange) which are used for management of exacerbations or occasionally for maintenance therapy in complex cases (Anderson et al., 2016; Gilhus and Verschuuren, 2015; Zinman et al., 2007). MG is a B-cell mediated disease, in that B-cells differentiate and proliferate into antibody producing plasma cells. MG is also T-cell dependent; CD4+ T-helper cells and T-regulatory cells facilitate the B-cell differentiation (Milani et al., 2006). The thymus gland is the primary site of production of autoantibodies in MG (Weiss et al., 2013). Thymectomy has become part of the standard of care for MG patients with a known thymoma. In addition, a randomised control trial in 2016 comparing thymectomy and prednisolone vs prednisolone alone in non-thymomatous AChR-MG also showed that thymectomy resulted in clinical benefit, as measured by a reduced quantitative myasthenia gravis (QMG) score (Wolfe et al., 2016).

This provides evidence for thymectomy in all patients with AChR-MG regardless of whether thymoma is present on imaging.

Acetylcholinesterase inhibitors (AChEIs) such as pyridostigmine, are recommended as part of the initial treatment for all AIMG subtypes (Sanders et al., 2016). Treatment results in rapid but usually incomplete symptomatic benefit. For some patients who have purely ocular symptoms and no generalised weakness treatment with AChEIs alone may be adequate. Different guidelines recommend varying escalation protocols for commencing AChEIs. The Association of British Neurologists guidelines are: 30mg pyridostigmine four times a day for 2-4 days; 60mg pyridostigmine four times a day for 5 days; then 90mg four times a day if required (Sussman et al., 2015).

Corticosteroids like prednisolone are used as short-term immunosuppressants in MG. Patients may take up to 3 months to respond. In addition, at around 4-10 days after starting prednisolone, a proportion of MG patients experience exacerbation of their myasthenic weakness, known as the “steroid dip” (Warmolts and Engel, 1972). For this reason it is recommended that steroids are commenced at a low dose on alternate days (e.g. 10mg once daily), and gradually titrated up (e.g. increase by 10mg every 3 doses until symptoms improve) (Sussman et al., 2015).

When symptom control is not achieved with corticosteroids and prednisolone, a steroid sparing immunosuppressant agent should be introduced. The first line agent is azathioprine, which is very slow to take its maximal effect (up to 2 years) (Palace et al., 1998). If azathioprine fails or is not tolerated, other immunosuppressive agents can be used. These include mycophenolate mofetil, methotrexate, ciclosporin, cyclophosphamide and rituximab (Sussman et al., 2015). Whilst these have been shown to be clinically effective in AIMG, they are also associated with serious side effects including nephrotoxicity, malignancy and bone marrow suppression (Sathasivam, 2011).

The immunomodulatory therapies intravenous immunoglobulin (IVIG) and plasma exchange are frequently used for management of MG relapses or occasionally as maintenance therapy. IVIG has demonstrated clear benefit in randomised controlled trials (Zinman et al., 2007). In general, for many aspects of the management of MG, evidence is lacking. In

addition, individuals with MG show a high degree of clinical variability and subsequently treatment is often tailored on an individual basis.

In Lambert Eaton Myasthenic Syndrome (LEMS) antibodies to presynaptic P/Q-type voltage-gated calcium channels (VGCCs) are detected in over 90% of patients (Eaton and Lambert, 1957; Motomura et al., 1995). Approximately 60% of LEMS cases are associated with malignancy, usually small cell lung cancer. Overlapping clinical features to MG can lead to diagnostic delay (Pellkofer et al., 2009). In addition to fatigable proximal weakness, patients present with autonomic symptoms and diminished tendon reflexes (Titulaer et al., 2011). The diagnosis is based on the finding of a distinctive electrophysiological pattern on repetitive nerve stimulation; the baseline compound muscle action potential (CMAP) is small, but increases >100% (increment) following high frequency stimulation or exercise (Oh et al., 2005). Approximately 10-15% of LEMS patients are seronegative (Titulaer et al., 2011). Symptomatic improvement for LEMS is obtained with administration of 3,4-diaminopyridine (3,4-DAP). 3,4-DAP inhibits presynaptic voltage-gated potassium channels, prolonging presynaptic VGCCs opening time (Kirsch and Narahashi, 1978).

1.2.2 Toxins and the NMJ

In addition to autoantibody attack, there are many toxins which in particular target the NMJ either presynaptically (e.g. tetrodotoxin, botulinum toxin, β-bungarotoxin, α-latrotoxin) or postsynaptically (e.g. D-tubocurarine, α-bungarotoxin). In addition to their role in disease and therapeutics, the study of these toxins has provided insights into the ability of the NMJ to adapt and recover from changing environments in order to maintain reliable neurotransmission (Slater, 2008). Most environmental compounds act by inhibiting AChE, including organophosphates and sarin (Abou-Donia and Lapadula, 1990; De Bleecker et al., 1994). Blocking the action of AChE leads to repeated opening of AChRs, depolarisation of the postsynaptic membrane, and inactivation of postsynaptic $Na_v1.4$ channels and the loss of ability to generate a muscle action potential. The botulinum neurotoxins are incredibly potent presynaptic toxins which cleave components of the SNARE complex, preventing ACh release from the nerve terminal (Schantz and Johnson, 1992). Intoxication causes flaccid paralysis and respiratory failure which can be fatal (Schantz and Johnson, 1992). They are

also valuable therapeutic agents, used for the treatment of spasticity and dystonia, resulting in temporary paralysis of selected muscles (Johnson, 1999).

1.2.3 The NMJ in disorders of the motor nerve

1.2.3.1 The NMJ in spinal muscular atrophy

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disease with an incidence of 1 in 10,000 live births, caused by mutations in the survival motor neuron gene (*SMN1*) (Prior et al., 2010; Verhaart et al., 2017). The centromeric homolog *SMN2* can generate a small amount of functional SMN protein, accounting for the four main clinical subtypes (SMA type I-IV) which vary in severity (Bürglen et al., 1996; Finkel et al., 2015). The SMN protein is ubiquitously expressed, but *SMN* mutations lead to selective loss of lower motor neurons. The SMN protein plays an essential role in regulating RNA metabolism, actin cytoskeleton dynamics, mRNA transport, ubiquitin homeostasis and synaptic vesicle release (Donlin-Asp et al., 2016; Groen and Gillingwater, 2015; Hensel and Claus, 2018; Kong et al., 2009; Li et al., 2014). However, it is still not precisely known how a reduction in SMN leads to death of motor neurons.

Several lines of investigation have sought to determine whether retrograde signals from the NMJ may contribute to the loss of motor neurons in SMA. Numerous studies have shown early morphological NMJ abnormalities in the SMNΔ7 SMA mouse, which precede the loss of motor neurons. These include smaller nerve terminals, reduced synaptic vesicle density, delayed switch of the AChR subunit from γ to ε, reduced postsynaptic area and simplified end-plates (reduced number of perforations per end-plate) and neurofilament accumulation (Boido et al., 2018; Kariya et al., 2008; Kong et al., 2009; Ling et al., 2012; Ruiz et al., 2010; Valsecchi et al., 2015). These findings were confirmed in post-mortem studies from SMA type 1 patients, which demonstrated small, non-perforated end-plates with prolonged expression of the γ-AChR subunit (Harding et al., 2015; Martínez-Hernández et al., 2013). In addition, in an *in vitro* model co-culturing motor neurons derived from induced pluripotent stem cells from SMA patients with murine C2C12 myoblasts, impaired AChR clustering and neurofilament accumulation was observed (Yoshida et al., 2015). These structural alterations are compounded by changes in NMJ function, including reduced quantal content

and intermittent failure of transmission in the SMNΔ7 SMA mouse (Kariya et al., 2008; Kong et al., 2009).

However, whether these abnormalities play a significant role in motor dysfunction in SMA is a topic of debate. More recent studies have shown impairment of spinal networks, including proprioceptive input, occurs well in advance of NMJ deficits in SMA mouse models (Mentis et al., 2011). Further studies in SMA mouse models and particularly in humans are required in order to elucidate the contribution of the NMJ to motor neuron loss in SMA. One study has shown that treatment of SMA mice with a soluble fragment of agrin (NT-1654) lead to improved motor function, reduction in NMJ morphological defects and increased muscle fibre area (Boido et al., 2018). These results indicate that the NMJ could represent a therapeutic target, at least for development of therapies to complement gene therapies and antisense oligonucleotide approaches.

1.2.3.2 The NMJ in amyotrophic lateral sclerosis

Motor neuron disease (MND)/Amyotrophic lateral sclerosis (ALS) is a fatal neuromuscular disease with degeneration of motor neurons leading to muscle weakness and wasting, paralysis, and respiratory failure (Paez-Colasante et al., 2015). The only approved ALS therapy, riluzole, does not modify the disease trajectory and prolongs life by a few months only (Lacomblez et al., 1996). Multiple pathological processes are implicated in ALS, including oxidative stress, abnormal energy metabolism, accumulation of protein aggregates, glutamate excitotoxicity, and glial dysfunction (Paez-Colasante et al., 2015). It remains in question whether motor neuron degeneration in ALS occurs through primary pathological insult in the motor cortex, and extends towards corticospinal neurons (dying forward), or whether degeneration begins at motor nerve terminals at the NMJ and progresses towards the anterior horn cell (dying back) (Cappello and Francolini, 2017). However, it has been shown that NMJ disassembly is a critical point in ALS pathogenesis.

The $SOD1^{G93A}$ mouse is a transgenic model which overexpresses human SOD1 (Gurney et al., 1994). This results in adult onset motor neuron degeneration with the first signs of muscle weakness becoming apparent at 4 months, and death occurring by 5-6 months of age (Gurney et al., 1994). NMJ structural alterations have been shown to be present in $SOD1^{G93A}$

mice before the onset of weakness or of motor neuron cell body loss (Fischer et al., 2004). In the SOD1^{G93A} mouse, early in the disease process, the postsynaptic membrane becomes fragmented, AChR density is reduced, some end-plates become denervated and some NMJs exhibit sprouting and collateral innervation (Valdez et al., 2012). In addition, ultrastructural analysis of NMJs of SOD1 mice showed mitochondrial alterations and reduced synaptic vesicle density in nerve terminals (Cappello et al., 2012). Presymptomatic NMJ disassembly has also been shown in canine ALS (Rich et al., 2002). The clinical relevance of the possible early and central role of the NMJ is the potential for treatment. A single dose of AAV *DOK7* gene therapy was shown to improve lifespan and motor activity and reduce muscle atrophy in the SOD1 mouse (Miyoshi et al., 2017).

Unfortunately, studies of the NMJ in the early stages of ALS in patients are very limited, due to the difficulty in obtaining muscle samples containing NMJs. Therefore, little is known about whether these structural defects also occur in humans, and what their functional implications might be on neuromuscular transmission. A few studies in patients have demonstrated that the postsynaptic architecture is not significantly altered in ALS, but nerve terminals are smaller (Tsujihata et al., 1984; Yoshihara et al., 1998). However, in a more recent study, which benefits from confocal fluorescence microscopy, significant postsynaptic morphological alterations were observed in all patients regardless of disease stage (Bruneteau et al., 2015). Only a single study has analysed NMJ function in ALS patients using intracellular electrophysiology (Maselli et al., 1993). This showed that mean quantal content of endplate potentials, the mean quanta available for immediate release, and the mean quantal stores were all decreased in ALS patients. However, it is not known whether these effects imply a primary NMJ defect or whether the reduced quantal content reflects NMJ denervation and axonal degeneration. Further functional studies will be important therefore, in understanding the clinical relevance of NMJ dismantling in ALS.

1.2.3.3 The NMJ in inherited motor neuropathies

Genetic neuropathies are a heterogenous group of inherited disorders affecting peripheral motor and/or sensory nerves. The most common form is Charcot–Marie–Tooth (CMT) disease, or hereditary motor sensory neuropathy (HMSN) in which both motor and sensory nerves are involved and which can be demyelinating (CMT1) or axonal (CMT2). With a

prevalence of 1 in 2500, CMT is one of the most common inherited neuromuscular diseases (Rossor et al., 2016). More than 80 genes are now known to be associated with CMT (Stojkovic, 2016). Although our understanding of the various biological processes that CMT genes are involved in has improved, there is still no effective treatment available for the majority of CMT (Corrado et al., 2016).

Several recent discoveries have implicated the NMJ in the pathogenesis of CMT. One recently discovered disease gene is *SYT2*, which encodes Synaptotagmin 2, a synaptic vesicle protein which functions as the main calcium sensor for neuromuscular transmission (Shields et al., 2017). Mutations in *SYT2* were identified in two large kinships with an autosomal dominant motor neuropathy syndrome and evidence of a presynaptic neuromuscular transmission defect on neurophysiological tests (see also section 1.3.2.1) (David N. Herrmann et al., 2014; Whittaker et al., 2015a). In addition, mutations in the presynaptic choline transporter CHT1, encoded by *SLC5A7*, have also been shown to cause an autosomal dominant motor neuronopathy (distal hereditary motor neuropathy type 7)(Barwick et al., 2012). These findings suggest that presynaptic NMJ dysfunction may be a more general mechanism for peripheral axonopathies.

NMJ defects have also been found in animal models of motor neuropathies. The $Gars^{c201r}$ mouse, a model of Charcot–Marie–Tooth Type 2D (CMT2D), which is caused by dominant mutations in Glycyl tRNA synthetase (*GARS*), exhibits smaller and fragmented end-plates which is independent of innervation status of the NMJs, and occurs prior to the loss of motor neuron connectivity (Sleigh et al., 2014; Spaulding et al., 2016). The *Cramping1 (Cra1/+)* mouse has hypomorphic mutations in *DYNC1H1*, which encodes the heavy chain of cytoplasmic dynein-1, a motor complex required for retrograde axonal transport. Mutations in *DYNC1H1* are implicated in a wide range of human neurological diseases, including CMT2O, and spinal muscular atrophy with lower extremity predominance (SMALED) (Niu et al., 2015, p. 1; Weedon et al., 2011). *Cra1/+* mice exhibit simplified end-plates with reduced pre and postsynaptic colocalisation early in the disease process prior to the loss of motor neurons (Courchesne et al., 2011). In the mouse model of CMT4C (Sh3tc2ΔEx1/ΔEx1 knockout), which is caused by recessive mutations in *SH3TC2*, NMJ morphological defects have been demonstrated, including increased fragmentation of end-plates and reduced area

of contact between pre- and post-synaptic components. In addition, the Sh3tc2ΔEx1/ΔEx1 knockout mice displayed prolonged expression of AChR γ subunit (Cipriani et al., 2018).

Using neurophysiological studies (repetitive nerve stimulation and single fibre electromyography), NMJ defects have been identified in a subgroup of CMT patients (Bansagi et al., 2017). Further studies will be aimed at characterising the role of the NMJ in CMT disease pathogenesis, and in assessing the therapeutic benefit of both novel and readily available therapies which enhance neuromuscular transmission.

1.3 Congenital Myasthenic Syndromes

The most recently identified conditions which arise from impairment of neuromuscular transmission are the congenital myasthenic syndromes (CMS). Although CMS are the rarest of the myasthenic disorders affecting man (estimated prevalence 1 per 100,000), they have nevertheless shown the greatest clinical and pathological diversity, and the study of CMS has expanded our knowledge both of how NMJ dysfunction can present clinically and of the role of previously uncharacterised proteins at the NMJ. CMS arise from mutations affecting crucial presynaptic, synaptic or postsynaptic proteins, resulting in impairment of the safety margin by one or more mechanisms (Engel et al., 2015a). The first identified mutations were in the subunits of the AChRs, and these remain the most common subtype of CMS worldwide today. However, in recent years and with the advent of next generation sequencing, the discovery of CMS related genes has accelerated and to date 31 genes have been implicated (Figure 8) (McMacken et al., 2017).

1.3.1 Clinical diversity in Congenital Myasthenic Syndrome

A generic diagnosis of a CMS is suggested on the basis of early onset (at birth to early childhood) of fatigable weakness in particular affecting the ocular and other cranial muscles. However, several pitfalls and challenges in diagnosis exist. CMS may first become evident during adolescent or adult life (Engel et al., 1982). In addition, unlike the fatigability in autoimmune MG, fatigability in CMS tends to fluctuate over a longer period, being week-to-week or month-to-month, rather than minute-to-minute, and thus may not be demonstrable on clinical exercise tests. Muscle weakness in some subsets of CMS may be predominantly proximal and axial with minimal involvement of cranial muscles, mimicking a limb-girdle muscular dystrophy (known as LG-CMS) (Evangelista et al., 2015). Early onset and a positive family history are also indicative, but their absence should not rule out the consideration of CMS as several subtypes may first become evident during adolescent or adult life (Engel et al., 1982). CMS may be particularly difficult to diagnose in neonates in whom non-specific features such as poor suck or cry, generalised hypotonia or arthrogryposis may be the only clinical features. A candidate gene may be indicated by

certain clinical, histopathological and neurophysiological findings (Table 1), although a subset of clinical CMS patients remain genetically undefined (estimated 10% in UK population) (McMacken et al., 2017).

Neurophysiological confirmation of a defect in neuromuscular transmission is an important step towards securing a diagnosis of CMS. The main tests used are repetitive nerve stimulation (RNS) and single-fibre electromyography (SFEMG). RNS involves stimulation of the motor nerve via surface electrodes and measurement of the resulting summated response, the CMAP (Whittaker, 2011). In patients with impaired neuromuscular transmission, the CMAP amplitude can vary with repetitive stimulation, with a reproducible percentage change (decrement) of 10% or more between the 1st and 4th CMAP amplitude being indicative of a NMJ disorder. In other presynaptic CMS subtypes in which the defect is not in impairment of ACh release, but in its synthesis in the presynaptic nerve terminal (e.g. due to mutations in *CHAT*, *SLC5A7* and *SLC18A3*), often no decrement is detected at low frequency (3Hz) stimulation, and it isn't until the pool of synaptic vesicles has been exhausted that any decrement is observed. Thus in such cases, prolonged high frequency RNS (e.g. 10Hz for 5 minutes) is necessary to ensure the diagnosis is not overlooked (Byring et al., 2002; McMacken et al., 2018; Mora et al., 1987). A "double CMAP" response should lead to consideration of slow channel CMS and ColQ-CMS, and is caused by generation of a second action potential within a muscle fibre following only a single motor nerve stimulation (van Dijk et al., 1996).

SFEMG is a more sensitive test and can detect milder defects of NMT, as it measures the variability (or "jitter") in the time taken to excite the muscle fibre. When normal in a weak muscle, SFEMG can exclude the diagnosis of an NMJ defect with some certainty. However, SFEMG is not a specific test for NMJ dysfunction as increased jitter can be found in the early stages of reinnervation and in myopathies (e.g. mitochondrial myopathy), and will increase slightly with age (Bromberg and Scott, 1994; Cruz-Martínez et al., 2004; Whittaker, 2011). Any neurophysiological evidence of an NMJ defect therefore must be interpreted in the context of clinical symptoms and signs.

All patients with suspected CMS and negative family history should have confirmation of a lack of antibodies to AChR or MuSK. Serum creatine kinase (CK) is typically normal in CMS, apart from CMS due to mutations in *GMPPB* in which the serum CK can be elevated 10 times

higher than normal (Rodríguez Cruz et al., 2016). Muscle MRI and muscle biopsy may be useful particularly in confirming a lack of major muscle pathology despite significant clinical weakness (Finlayson et al., 2016). An exception is MRI in CMS due to glycosylation defects (see below) in which non-specific fatty change can be observed. Most CMS subtypes display minimal myopathic features on muscle biopsy. These may be more marked in Dok7 CMS, ColQ CMS and slow channel syndrome (Engel et al., 1982; Hutchinson et al., 1993; Selcen et al., 2008). In CMS due to glycosylation defects, specifically *DPAGT1* and *GFPT1*, tubular aggregates may be seen on NADH staining (Belaya et al., 2012; Guergueltcheva et al., 2012).

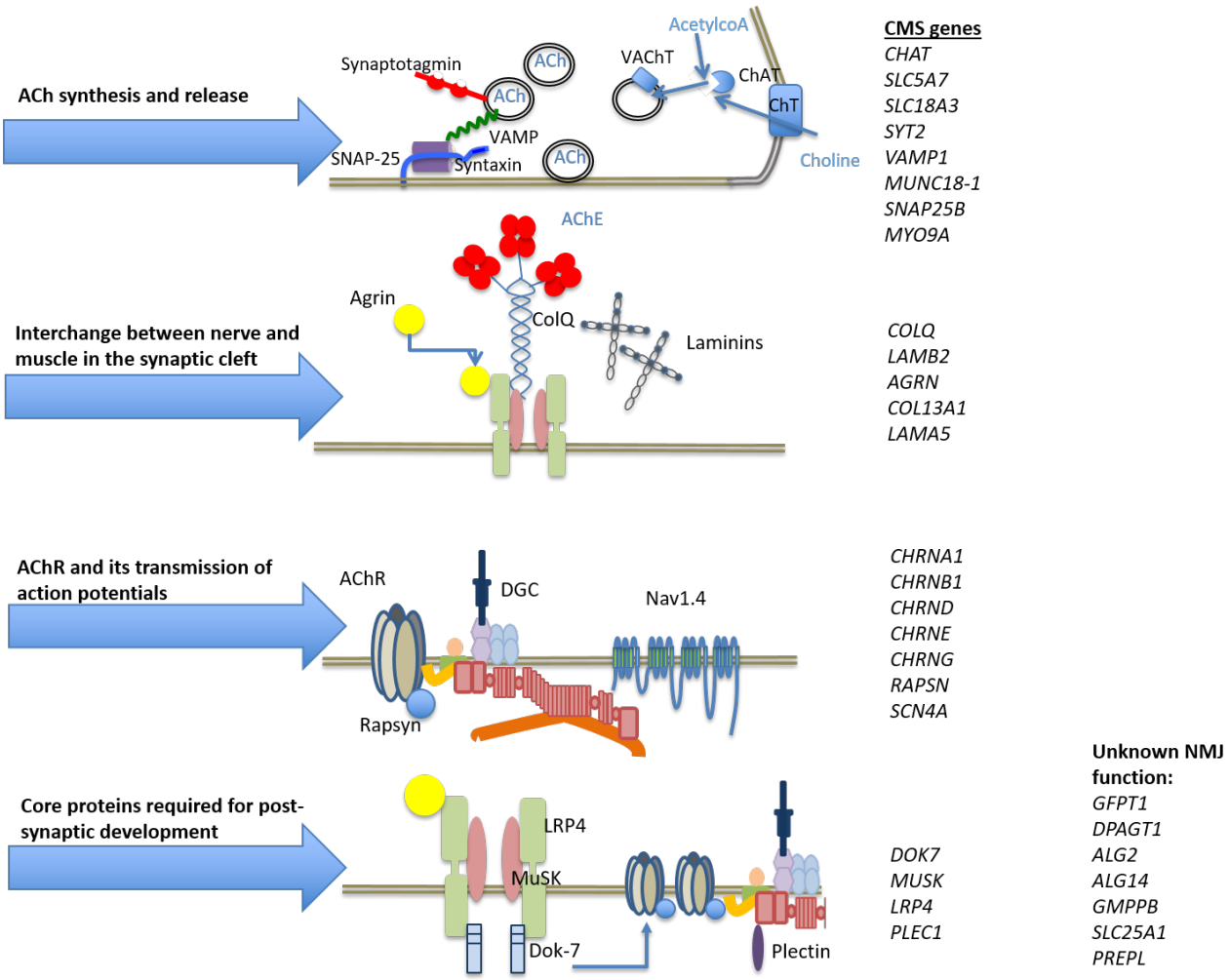

Figure 8: Genetic subtypes of congenital myasthenic syndrome. Genes can be grouped according to their role in the development and function of the NMJ, although for a subset of these genes their specific role in the development of CMS is unknown. ACh: acetylcholine; AChE: acetylcholinesterase; AChR: acetylcholine receptor; ChT: high affinity choline transporter, DGC: dystrophin associated glycoprotein complex, Nav1.4: voltage gated sodium channel. Adapted from McMacken et al 2017.

Gene	*Protein and function*	*Key Clinical features*	*Treatment*
Genes influencing ACh synthesis and release			
CHAT	Choline acetyltransferase (ChAT); resynthesis of ACh from choline and acetyl-CoA in the presynaptic nerve terminal.(Oda, 1999)	Early onset CMS-EA. Mild clinical course between symptoms with varying degrees of progressive proximal weakness both between and within families.	Pyridostigmine
SLC5A7	Presynaptic high affinity choline transporter 1 (CHT1); ACh re-uptake into synaptic vesicles	Severe CMS of early onset. Presentations include a severe prenatal form resulting in arthrogryposis, hypotonia and early lethality, to neonatal onset CMS-EA	Pyridostigmine
SLC18A3	Vesicular acetylcholine transporter (VAChT); concentration of ACh in presynaptic vesicles.	Patients present with respiratory crises, ptosis, ophthalmoplegia and fatigability. Deterioration of symptoms in cold temperature described in one case.	Pyridostigmine
MYO9A	Myosin9A; unconventional myosin with presumed pre-synaptic origin.	Described in three cases of neonatal onset hypotonia with bulbar and ocular involvement, as well as respiratory crises with EA.(O'Connor et al., 2016)	Pyridostigmine Adjunctive therapy with 3,4-DAP
SYT2	Synaptotagmin 2; main calcium sensor for neurotransmission.	Motor axonopathy syndrome, with some fatigability on examination and neurophysiological features of a presynaptic myasthenic syndrome, similar to LEMS (David N. Herrmann et al., 2014)	3,4-DAP
SNAP25B	SNAP25 (synaptosomal-associated protein of 25 kD); forms part of SNARE complex, required for regulation of synaptic vesicle fusion(Chen et al., 2002)	Severe CMS of neonatal onset, with associated cortical excitability, intellectual disability and cerebellar ataxia.(Shen et al., 2014, p. 2)	3,4-DAP
MUNC13-1	Munc13-1; associates with the SNARE complex to regulate docking and priming of synaptic vesicles.(Augustin et al., 1999; Betz et al., 1998; Siksou et al., 2009)	Described in one case, causing a severe syndrome of neonatal onset with hypotonia, respiratory distress, ptosis and ophthalmoplegia, as well as facial dysmorphism, microcephaly and abnormal cortical electrical activity.(Engel et al., 2016)	Pyridostigmine and 3,4-DAP (limited benefit)
VAMP1	Synaptobrevin 1; component of the SNARE complex required for synaptic vesicle fusion(Adams et al., 2015)	A homozygous frameshift mutation in *VAMP1* is described in a case of severe neonatal CMS with marked hypotonia, with respiratory and oculomotor involvement.(Shen et al., 2017)	Pyridostigmine (modest improvement)
Genes influencing the interchange between nerve and muscle in the synaptic cleft			
COLQ	ColQ; collagenic-like strand responsible for anchoring AChE to the synaptic basal lamina	Broad phenotype ranging from adult onset LG-CMS, to the early onset severe and progressive forms.(Mihaylova et al., 2008; Shapira et al., 2002) Slowing of pupillary light reflex in 25% of cases.	Salbutamol or ephedrine Worsening with pyridostigmine

LAMB2	Laminin β2; component of the synaptic basal lamina of the NMJ.(Noakes et al., 1995)	Mutations in *LAMB2* are well described in Pierson syndrome, which causes congenital nephrosis and ocular abnormalities.(Zenker et al., 2004) LAMB2 mutations have also been found in one patient who, in addition to the renal and ocular manifestations, presented with a CMS with ptosis, respiratory insufficiency and proximal limb weakness.	Ephedrine Worsening with pyridostigmine
AGRN	Agrin; nerve-derived component of the basal lamina which induces AChRs to cluster on the post-synpatic membrane.(McMahan, 1990)	*AGRN* mutations are a relatively rare cause of CMS, and may cause ptosis, fatigable proximal limb weakness and respiratory insufficiency.(Huzé et al., 2009; Maselli et al., 2012) *AGRN* mutations are also described in cases of CMS with distal weakness and features in-keeping with a distal myopathy on MRI.(Nicole et al., 2014)	Salbutamol or ephedrine
COL13A1	α-chain of the muscle derived collagen 13; embedded in the ECM and plays a role in NMJ maturation.(Latvanlehto et al., 2010)	*COL13A1* mutations cause CMS bearing some resemblance to the more common CMS due to Rapsyn deficiency, with neonatal onset, dysmorphic features, ptosis and respiratory involvement.(Logan et al., 2015)	Ephedrine Adjunctive therapy with 3,4 DAP
LAMA5	Laminin α5; binds to the luminal side of synaptic vesicles(Rogers and Nishimune, 2017)	Severe early onset with CNS involvement.(Maselli et al., 2017)	Pyridostigmine Adjunctive therapy with 3,4-DAP
Genes affecting the AChR and its transmission of action potentials			
AChR deficiency (*CHRNE)*	ε-subunit of the pentameric AChR	The most common form of CMS. Clinically variable but typically ophthalmoplegia, ptosis, dysphagia and fatigable proximal muscle weakness with onset in early infancy.	Pyridostigmine Adjunctive therapy with salbutamol or ephedrine
AChR deficiency (*CHRNA1/ B1/D)*	α, β and δ subunits of the AChR	Not as common AChR deficiency due to *CHRNE*, but often more severe(Engel et al., 2015b; Gomez et al., 2002; Shen et al., 2016), with arthrogryposis, dysmorphism, scoliosis and respiratory involvement.	Pyridostigmine Adjunctive therapy with salbutamol or ephedrine
RAPSN	Rapsyn; scaffolding protein, interacts with AChRs to induce clustering.(Gautam et al., 1995)	Typically early-onset with life-threatening apneic crises from birth, hypotonia, arthrogryposis multiplex congenital and feeding difficulties.(Burke et al., 2003) Later onset milder form also occurs.	Pyridostigmine
Fast channel syndrome	Altered kinetic properties of the AChR, so as to cause an inappropriately fast closure of the channel following binding of ACh.(Ohno et al., 1996)	Severe myasthenic weakness with respiratory crises, with onset in early infancy or childhood(Webster et al., 2014)	Pyridostigmine Adjunctive therapy with 3,4-DAP

Slow channel syndrome	Prolonged opening of the AChR following ACh binding	Usually onset in late childhood, often selective involvement of finger flexors and neck extensors and no extraocular involvement.	Fluoxetine or quinidine Adjunctive therapy with salbutamol or ephedrine
SCN4A	Voltage gated sodium channel ($Na_v1.4$); allow influx of sodium ions into postsynaptic membrane and generation of a muscle action potential	More commonly associated with non-dystrophic myotonia. However, mutations have also been described in three unrelated cases of CMS.(Arnold et al., 2015; Habbout et al., 2016; Tsujino et al., 2003) Relatively severe limb weakness, ophthalmoplegia and ptosis are described in all cases.	Pyridostigmine
Genes affecting the core proteins required for post-synaptic development			
DOK7	Dok-7; cytoplasmic adaptor protein which activates MuSK and is essential for normal maturation of the post-synaptic membrane.(Yamanashi et al., 2008)	LG-CMS with waddling gait, tongue wasting +/- ptosis. Typically onset in late childhood but variable. Varying degrees of respiratory involvement. Respond well to sympathomimetics	Salbutamol or ephedrine Worsening with pyridostigmine
MUSK	Muscle specific receptor tyrosine kinase (MuSK); interacts with a large number of effectors and pathways converging toward the regulation of AChR clustering and postsynaptic specialisation.	Ranging from severe phenotype, respiratory insufficiency at birth and proximal muscle weakness to later onset LG-CMS.(Mihaylova et al., 2009; Owen et al., 2018)	Salbutamol or ephedrine Adjunctive treatment with 3,4-DAP
LRP4	LRP4; transmembrane post-synaptic protein which triggers NMJ development when bound to by agrin.(Kim et al., 2008)	Mutations in *LRP4* have been identified in Cenani-Lens syndactyly syndrome, bone overgrowth and low bone mineral density,(Leupin et al., 2011; Li et al., 2010, p. 4; Styrkarsdottir et al., 2009) but are a very rare cause of CMS. Few cases described exhibit a variable phenotype, from a severe neonatal presentation with hypotonia, ptosis and ophthalmoplegia, to a progressive LG-CMS.(Ohkawara et al., 2014, p. 4; Selcen et al., 2015, p. 4)	Worsening with pyridostigmine
PLEC1	Plectin; intermediate filament-associated protein concentrated at sites of mechanical stress, including Z-discs in muscle.(Maselli et al., 2011)	Pathogenic mutations in *PLEC1* are associated with neonatal epidermolysis bullosa simplex, and, in some cases, with a muscular dystrophy and a defect of neuromuscular transmission.(Forrest et al., 2010)	Ephedrine Pyridostigmine no/limited benefit
Genes affecting ubiquitous proteins with unknown NMJ function			
GFPT1	Required for synthesis of the rate limiting enzyme for N- and O-linked glycosylation	LG-CMS with minimal extraocular involvement. Tubular aggregates on muscle biopsy. Onset in late childhood or adulthood	Pyridostigmine Adjunctive therapy with 3,4-DAP

DPAGT1	Encodes the enzyme which catalyses the first step of N-linked glycosylation	LG-CMS with minimal extraocular involvement. Tubular aggregates on muscle biopsy. Onset in late childhood or adulthood	Pyridostigmine Adjunctive therapy with 3,4-DAP
ALG2 and *ALG14*	Encodes enzymes which catalyse early steps in N-linked glycosylation	LG-CMS with ptosis but minimal ophthalmoplegia of onset in childhood. Joint laxity and pes planus also described.	Pyridostigmine
GMPPB	Required for O-glycosylation of α-dystroglycan	LG-CMS of late childhood or adult onset. Uniquely elevated CK (10 times higher than normal). Mutations also associated with congenital muscular dystrophy with generalised epilepsy.(Raphael et al., 2014)	Pyridostigmine Adjunctive therapy with salbutamol or ephedrine
SLC25A1	Encodes the mitochondrial citrate transporter, responsible for the movement of citrate across the mitochondrial inner membrane.	Varying presentations from a severe mitochondrial disease with cardiac, eye and brain involvement, to less severe CMS.(Chaouch et al., 2014; Edvardson et al., 2013) The reason for mutations in a ubiquitous mitochondrial citrate carrier manifesting primarily as a NMJ defect remains to be determined.	3,4-DAP
PREPL	Encodes the enzyme Prolyl endopeptidase-like, which is expressed in brain, kidney and muscle.(Régal et al., 2014)	PREPL deficiency in combination with other deletions causes the hypotonia cystinuria syndrome, characterised by neonatal hypotonia, cystinuria, growth hormone deficiency and feeding problems.(Parvari et al., 2001) PREPL deficiency is also described in one case of a neonatal CMS without cystinuria.(Régal et al., 2014)	Pyridostigmine (transient response)

Table 1: Congenital Myasthenic Syndrome subtypes and treatment response. Definitions: ACh – acetylcholine; AChE – acetylcholinesterase; AChEI – acetylcholinesterase inhibitor; AChR – acetycholine receptor; CMS – congenital myasthenic syndrome; CMS-EA – congenital myasthenic syndrome with episodic apnea; LG-CMS – limb-girdle congenital myasthenic syndrome; NMJ – neuromuscular junction. Adapted from McMacken et al 2017.

1.3.2 Genetic heterogeneity in CMS

Genetic diagnosis is of paramount important in CMS because it allows the administration of tailored treatment, as well as providing prognostic information and facilitating genetic counselling. Single gene testing may be carried out on a gene-after-gene basis if certain clinical or laboratory findings indicate a specific subtype, although multi-gene panel testing is becoming frequently used as a first-line investigation. When single gene or panel testing fail to identify the genetic defect, whole genome or whole exome sequencing may be considered. Apart from the slow channel syndrome and *SYT2* CMS which are inherited in an autosomal dominant manner, all CMS are autosomal recessive.

Genes of particular interest and recent developments of some CMS genetic subtypes are discussed below.

1.3.2.1 Novel presynaptic genes

Until very recently, almost all CMS subtypes were due to defects in the postsynaptic apparatus or the synaptic cleft. The last three years have seen a rapid increase in the number of presynaptic CMS genes. These encode proteins involved in processes related to ACh re-uptake and recycling, and ACh vesicular release; (*CHAT*, *SLC5A7* and *SLC18A3*) and (*SYT2*, *VAMP1*, *SNAP25,* and *MUNC13-1*) respectively (Figure 9). Two additional genes are also thought to have a presynaptic role; *SLC25A1* encoding the mitochondrial citrate carrier and *MYO9A* (Chaouch et al., 2014, p. 25; O'Connor et al., 2016). Presynaptic CMS subtypes are often more severe, and may be associated with intellectual disability and behavioural problems, reflecting their role at cholinergic neurons in the CNS.

ACh re-uptake and recycling

The first pre-synaptic gene identified as causative for CMS was *CHAT*, which encodes the enzyme choline acetyltransferase (ChAT). ChAT is essential for the resynthesis of acetylcholine (ACh) from choline and acetyl-CoA in the presynaptic nerve terminal (Oda, 1999). In ChAT-CMS, recessive loss-of-function mutations impair the catalytic efficiency of the enzyme and decrease the release of ACh during physiologic activity (Arredondo et al., 2015). The clinical course between crises may be mild with minimal symptoms (Ohno et al., 2001), however long-term follow-up in these patients has shown that progressive limb weakness despite appropriate therapy may be a feature (Schara et al., 2010). *SLC5A7*

encodes the presynaptic high affinity choline transporter 1 (ChT), which was previously identified as the causative gene in distal hereditary motor neuronopathy type 7 (Barwick et al., 2012). Mutations in *SLC5A7* have also been identified in CMS, with 11 published cases to date (Bauché et al., 2016b). The presentations range from a severe form resulting in arthrogryposis, hypotonia and early lethality, to neonatal onset CMS with EA. *SLC18A3* encodes the vesicular associated choline transporter (VAChT) which packages newly synthesised ACh into synaptic vesicles. Recessive mutations are associated with a severe CMS with arthrogryposis and respiratory failure (Aran et al., 2017; O'Grady et al., 2016). A degree of positive response to AChEIs is seen in most cases with mutations in *CHAT*, *SLC5A7* or *SLC18A3.*

Genes involved in ACh synthesis and release are particularly associated with episodic apnoea (CMS-EA), recurrent periods of respiratory arrest emerging in the neonatal period or during the first months of life, and often resolving with age (Schara et al., 2010). Sudden recurrent apnoeic episodes are one of the most poorly understood features of CMS, and result in significant morbidity and mortality. The mechanism underlying these recurrent attacks is not known. The fact that this phenomenon occurs predominantly in presynaptic genes and in patients with a complex phenotype with CNS involvement suggests it may be a centrally mediated mechanism (Bauché et al., 2016a; McMacken et al., 2018).

CMS-LEMS

In CMS subtypes in which the primary defect results in impairment of ACh release, electrophysiological signatures which were previously felt to be diagnostic for Lambert Eaton Myasthenic Syndrome (LEMS) have been demonstrated. In these subtypes the baseline CMAP amplitude is low, and exhibits decrement on low-frequency RNS. However, RNS carried out at high frequency (e.g. 50Hz for 10 seconds) results in an opposing effect and an increment in CMAP amplitude is observed (Engel et al., 2016; Shen et al., 2017; Whittaker et al., 2015b). High frequency stimulation increases presynaptic calcium influx, saturating the calcium dependent release machinery and subsequently transiently increasing ACh release (Whittaker et al., 2015b). To date, the genes that have been associated with CMS–LEMS are *AGRN*, *SYT2*, *MUNC13-1*, *VAMP1*, and *LAMA5*, and this finding has shed new light on the role of these proteins in calcium-triggered ACh vesicle release at the NMJ (Lorenzoni et al., 2018).

SYT2 encodes Synaptotagmin 2, a presynaptic vesicle protein which functions as the main calcium sensor for neurotransmission. Mutations in *SYT2* were identified in families with a non-progressive motor axonopathy, with some patients showing subtle fatigability on examination (David N. Herrmann et al., 2014). This is the second identified CMS subtype which is inherited in an autosomal dominant fashion, and its discovery has expanded the role of the NMJ in hereditary motor axonopathies.

LAMA5 encodes laminin α5, an ECM protein which binds to synaptic vesicles after they have fused with the presynaptic membrane (Son et al., 2000). Despite the fact that it is ubiquitously expressed, mutations in *LAMA5* have been associated with a syndrome limited to a severe presynaptic CMS with underdeveloped nerve terminals (Maselli et al., 2017).

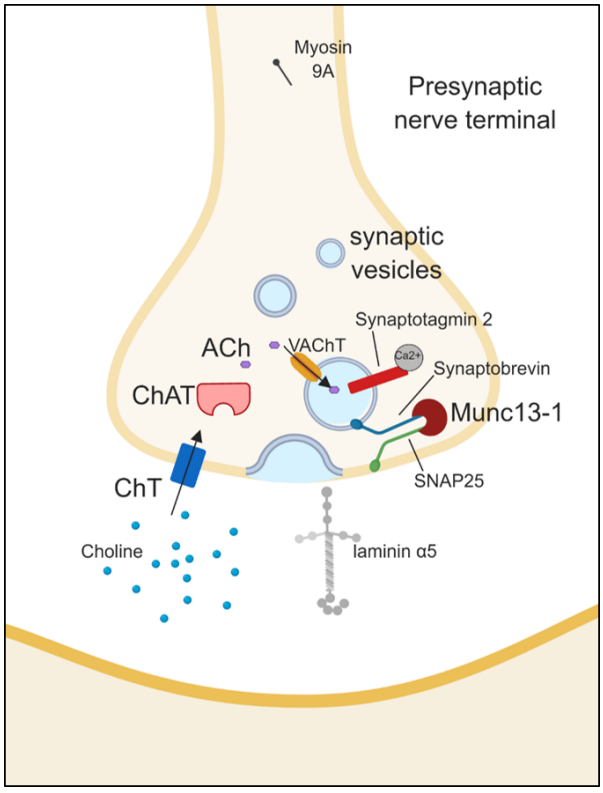

Figure 9: The presynaptic proteins at the NMJ implicated in congenital myasthenic syndromes. Synaptotagmin 2, synaptobrevin, SNAP25 and Munc13-1 are involved in synaptic vesicle release. Laminin alpha 5 binds to synaptic vesicles after fusion with the presynaptic membrane. ChAT, ChT and VAChT are instead involved in the re-uptake and recycling of choline from the synaptic cleft. Myosin 9A contributes to vesicle trafficking and agrin secretion. ChAT, choline acetyltransferase; ChT, high-affinity presynaptic choline transporter; SNAP25, synaptosomal-associated protein 25; VAChT, vesicular acetylcholine transporter.

1.3.2.2 Genes of the extracellular matrix

CMS caused by mutations in the *COLQ* gene have a broad phenotype ranging from adult onset limb girdle myasthenia which may be difficult to distinguish from a limb girdle muscular dystrophy, to the early onset severe and progressive forms (Mihaylova et al., 2008; Shapira et al., 2002). Uniquely to this CMS subtype, patients may exhibit slowing of the pupillary light reflex, which may serve as a diagnostic clue, but is only present in around 25% of cases (Mihaylova et al., 2008). In addition, a recent study has shown attenuated heart rate variability in ColQ patients, suggesting some degree of autonomic dysfunction (Günbey et al., 2019).

COL13A1 encodes Collagen XIII, which is ubiquitously expressed but concentrated at the NMJ, and stabilises AChR clusters (Latvanlehto et al., 2010). Patients with recessive mutations in *COL13A1* present with severe CMS, respiratory crises, non-fatigable ptosis and axial weakness (Beeson et al., 2018; Dusl et al., 2019; Logan et al., 2015, p. 1). This is a recently identified CMS subtype, but the emerging phenotype appears to be homogenous, which may facilitate earlier diagnosis in these patients (Dusl et al., 2019).

1.3.2.3 Postsynaptic CMS

Dok7-CMS is the second most common subtype of CMS in the UK. Patients with mutations in *DOK7* frequently present in late childhood, but presentations in adulthood or in infancy are well recognised. The spectrum of Dok7 CMS ranges from severe neonatal hypotonia with stridor, to adult onset LG-CMS with a classical waddling gait. In addition, wasting of tongue muscles may serve as a diagnostic clue (Cossins et al., 2012; Selcen et al., 2008; Yamanashi et al., 2008). The exon 7 frameshift duplication c.1124_1127dupTGCC is commonly found in at least one allele in these patients (Cossins et al., 2012). Analysis of motor point biopsies from Dok-7 patients demonstrate that their motor nerve terminals and motor endplates are significantly smaller than controls (Slater et al., 2006).

Closely related to Dok7 CMS is CMS due to mutations in Dok7's signalling partner *MUSK.* Mutations in *MUSK* were previously thought to be a very rare cause of CMS, and associated with a severe early onset disease (Mihaylova et al., 2009; Wilbe et al., 2015). However, through the use of next generation sequencing, a less severe adult-onset phenotype is now recognised, which overlaps with the LG-CMS phenotype of Dok7 CMS (Owen et al., 2018).

1.3.2.4 CMS caused by disorders of glycosylation

Glycosylation of nascent peptides is an essential post-translational modification throughout many biological processes (Krištić and Lauc, 2017). It follows therefore, that the congenital disorders of glycosylation are multisystem disorders which present in infancy. However, in contrast to this phenotype, 5 CMS causing genes have been identified which result in impairment of glycosylation; *GFPT1*, *DPAGT1*, *ALG2*, *ALG14* and *GMPPB* (Ohno, 2013). The role of glycosylation in CMS was first outlined following the discovery of mutations in *GFPT1* and *DPAGT1* in patients with LG-CMS, with limited involvement of facial or extraocular muscles. *GFPT1* is involved in the synthesis of the rate limiting enzyme required for *N*- and *O*-linked glycosylation, whilst *DPAGT1* encodes the enzyme which catalyses the first step of *N*-linked glycosylation (Belaya et al., 2012; Senderek et al., 2011). Patients exhibit several features overlapping with a myopathy, including myopathic features on EMG (in addition to decrement on RNS), fatty infiltration on MRI, and mildly elevated CK in some (Belaya et al., 2012; Finlayson et al., 2016; Guergueltcheva et al., 2012). Two further identified genes *ALG2* and *ALG14* are involved specifically in *N*-linked protein glycosylation. Affected patients exhibit limb-girdle weakness, mild ptosis, and minimal ophthalmoplegia, with onset in late childhood. The 5th identified glycosylation gene in CMS patients, *GMPPB*, had been previously shown to cause congenital muscular dystrophy due to hypoglycosylation of α-dystroglycan (Carss et al., 2013). Mutations in *GMPPB* were also identified as the cause of CMS with fatigable limb-girdle weakness presenting in late adolescence and adulthood, with the distinguishing feature of raised CK levels (Rodríguez Cruz et al., 2016). Like other CMS subtypes caused by glycosylation defects, these patients exhibited some myopathic features on both EMG and muscle biopsy (Rodríguez Cruz et al., 2016).

Many NMJ proteins are glycosylated, but the reason that defects in such key biological processes would lead to clinical syndromes restricted to NMJ dysfunction is unclear. The recent development of a mouse model of *GFPT1* CMS may aid in the understanding of to the pathomechanism of these disorders. This muscle specific knockout replicates many features of the human disease, suggesting the defect in these patients is primarily postsynaptic, and also identified dysregulated proteins in the agrin/MuSK pathway as a consequence of GFPT1 deficiency (Issop et al., 2018). Intracellular electrophysiological studies of NMJs in these

patients may also help to localise the site of the defect, although these have not been carried out to date.

1.3.3 Treatment of CMS

A precise molecular classification of CMS subtype is of great importance for the diagnosis and genetic counselling of patients, but also to allow administration of effective treatment as different drugs may be beneficial or deleterious depending on the CMS subtype (Engel et al., 2015a). Unlike many inherited neuromuscular disorders, most CMS subtypes are amenable to treatment with one or more symptomatic treatments (Table 1). However, for most of these treatments the mechanism of action is poorly understood or completely unknown. In addition, due to the genetic heterogeneity and rarity of CMS, high-level evidence on the efficacy and safety of these treatments is not available (Thompson et al., 2019).

1.3.3.1 Acetylcholinesterase inhibitors

Most CMS subtypes respond to AChE inhibitors (e.g. pyridostigmine) which augment the synaptic response to ACh (Schara and Lochmüller, 2008). The onset of action is rapid, and responsive patients will typically report functional benefit within days of starting the drug. Limb and bulbar weakness typically improve, but ptosis and ophthalmoplegia are often resistant to treatment. AChE inhibitors have a short duration of action and are given regularly throughout the day (e.g. four or five times per 24 hours); patients will often report mild worsening of symptoms when their next dose is due. The main side-effects are muscarinic (in particular diarrhoea, abdominal cramping and increased urination) and muscle fasciculations. In addition, inappropriately high doses may precipitate cholinergic crises.

1.3.3.2 3,4 Diaminopyridine

AChE inhibitors are often combined with 3,4 diaminopyridine (3,4-DAP) which blocks potassium channels in nerve terminals thereby prolonging action potential and increasing ACh release (Kirsch and Narahashi, 1978). 3,4-DAP has particular benefit in presynaptic subtypes. 3,4-DAP is well tolerated, with the most common side effect being mild

paraesthesia. However, 3,4-DAP can be associated with epileptic seizures, an adverse effect which is dose-dependent (Lindquist and Stangel, 2011).

1.3.3.3 Open Channel Blockers

Fluoxetine and quinidine are long-acting open channel blockers of the AChR. They are beneficial only in the treatment of slow channel syndrome (Fukudome et al., 1998; Harper et al., 2003). The open channel blockers act quickly to improve neuromuscular transmission, but there is also an increasing beneficial effect over time (e.g. 4 weeks), suggesting they may improve also improve the end-plate myopathy of slow channel syndrome (Harper and Engel, 1998). Fluoxetine is generally used first line due to a better safety profile (Engel, 2007). Quinidine can lead to a prolonged QT interval, and ECG monitoring is required.

1.3.3.4 Sympathomimetics

Ephedrine is a sympathomimetic amine with α- and β-adrenergic effects, which was first used in the treatment of autoimmune MG following the publication in the 1930s of case reports by Harriet Edgeworth, a physician who had noticed a dramatic improvements in her own myasthenic weakness when she began taking ephedrine (Edgeworth, 1933, 1930). The use of ephedrine in MG was subsequently superseded by the introduction of immunomodulating therapies and AChE inhibitors, and it wasn't until the discovery of CMS that the use of ephedrine in NMJ disorders was reintroduced. In countries where ephedrine is not easily available or in cases where ephedrine is not tolerated, salbutamol, a selective β_2 adrenoceptor (AR) agonist, has been successfully used to treat subsets of CMS.

Ephedrine or salbutamol are used in CMS subtypes where AChE inhibitors are ineffective or even detrimental, particularly end-plate AChE deficiency (*COLQ*) and Dok-7 CMS (Schara and Lochmüller, 2008; Slater et al., 2006). These have been successfully used to treat patients with CMS due to mutations in *AGRN, MUSK, LRP4* and glycosylation defects (Gallenmüller et al., 2014; Nicole et al., 2014; Salih et al., 2015; Selcen et al., 2008). In addition, salbutamol or ephedrine are increasingly used as an adjunctive therapy along with pyridostigmine in AChR deficiency (Cruz et al., 2015).

Whilst no randomised control trials have been carried out, owing to the rarity of these conditions, several case series and open label trials have confirmed the clinical benefit of

salbutamol and ephedrine (Table 2) (Thompson et al., 2019). Salbutamol and ephedrine appear to be equally efficacious, although again this has not been confirmed in clinical trials. Unlike the rapid clinical benefit seen with AChE inhibitors, the effect of salbutamol and ephedrine is a more gradual one (McMacken et al., 2017). Initial symptomatic benefit is observed within weeks, although peak effect may not be reached until 6 months of treatment (Engel, 2007). The typical adult starting dose of salbutamol is 4mg three times a day, and of ephedrine is 15mg three times a day. Doses should be titrated up according to clinical effect.

The main side-effects are muscle cramps, insomnia and cardiac effects (tachycardia, palpitations and hypertension). However, studies are lacking in the long-term effects of salbutamol and ephedrine in CMS, particularly in terms of cardiotoxicity (Thompson et al., 2019). In some cases, the clinical benefit from salbutamol may attenuate after years of treatment (unpublished observations). This may be due to desensitisation of β2-ARs after chronic agonist administration, as occurs when β2-AR agonists are used in the treatment of chronic heart failure and asthma. The development of more targeted therapies for CMS, which benefit muscle strength whilst minimising systemic side-effects is therefore essential to improve quality of care. In addition, an understanding of the effect of sympathomimetics at the NMJ is important in order to target treatment to the most appropriate patient groups. Furthermore, given the implication of the NMJ in CMT, ALS and SMA, pharmacological improvement of the NMJ will have wider applications beyond CMS.

1.3.3.5 Experimental CMS treatments

An engineered c-terminal fragment of agrin which is soluble (NT-1654) has been shown to be beneficial in mouse models of SMA and sarcopenia, and in NMJ recovery following nerve-crush (Boido et al., 2018; Hettwer et al., 2014). In addition, Dok7 gene therapy has been shown to be beneficial in a mouse model of Dok7 CMS (Arimura et al., 2014). Further experimental therapies are using repurposed drugs, including the antiepileptic zonisamide which increases nerve sprouting in mouse models (Ohno, 2017).

Publication	Dx	Subj	Drug	Length	Design	Efficacy
(Liewluck et al., 2011)	*COLQ* and *DOK7* CMS	18 (3,15)	Salbut	1-24m	Uncontrl	Significantly improved walking distance and no. of steps climbed (both groups)
(Burke et al., 2013)	*DOK7* CMS	9	Salbut	30m	Uncontrl	Improvements in timed tests, minimal observed functional benefits in ADL
(Gallenmüller et al., 2014)	*MUSK* CMS	2	Salbut	NA	Case report	Walking and stair climbing
(Lorenzoni et al., 2013)	*DOK7* CMS	5	Salbut	12m	Uncontrl	QMG score, 6mwt, ADL score
(Mihaylova et al., 2008)	*COLQ* CMS	5	Ephed	NA	Case report	Improved FVC and motor symptoms
(Bestue-Cardiel et al., 2005)	*COLQ* CMS	2	Ephed	24m	Case report	Improved motor symptoms
(Schara et al., 2009)	*DOK7* CMS	8	Ephed	12-24m	Uncontrl	Improved weakness (MRC scale)
(Owen et al., 2018)	*MUSK* CMS	4	Salbut	NA	Case series	Functional benefits in ADL
(Rodríguez Cruz et al., 2016)	*GMPPB* CMS	3	Salbut	NA	Case series	Improved motor symptoms
(Finlayson et al., 2013)	*DPAGT1* CMS	1	Salbut	NA	Case series	Improved MRC scale and motor symptoms
(Cruz et al., 2015)	*CHNRE* CMS	6	Salbut/ Ephed	8m	Uncontrl	QMG score, 10mwt

Table 2: Clinical Experience of β-Adrenergic Agonists in Congenital Myasthenic Syndrome. Definitions – 6mwt: 6 minute walk test, 10mwt: 10 minute walk test, ADL: activities of daily living, Dx: Diagnosis, Ephed: ephedrine, FVC: forced vital capacity, MRC: manual muscle testing using Medical Research Council scale, QMG: Quantitative myasthenia gravis score, Salbut: salbutamol, Subj: total subjects enrolled, uncontrl: uncontrolled; no placebo group.

1.4 Adrenergic Signalling and its role at the NMJ

1.4.1 Adrenoceptors and the Sympathetic Nervous System

The sympathetic nervous system (SNS) is one of the two main divisions of the autonomic nervous system, with the other being the parasympathetic nervous system, which serve to regulate involuntary reactions to stressors throughout the body tissues (Clar and Sharma, 2019). The two major chemical transmitters in the SNS are adrenaline and noradrenaline. These bind to adrenergic receptors to illicit a variety of responses, depending on the receptor subtype bound (Lynch and Ryall, 2008). Noradrenaline is the predominant neurotransmitter at sympathetic nerves. Noradrenaline is stored in synaptic vesicles in sympathetic nerve terminals and is released in response to sympathetic nerve impulses, and subsequently diffuses across the synapse to the effector organ. Adrenaline is a circulating neurohumoral factor which is synthesized and released from by the adrenal medulla. It is carried to local effector organs by the circulation (Stamper et al., 2009).

All adrenergic receptors are guanine nucleotide-binding G protein-coupled receptors (GPCRs) (Haga et al., 1994). G proteins are made up of the G_{α} subunit and the G_{β} and G_{γ} subunits, which form a stable dimeric complex (the $G_{\beta\gamma}$ complex) (Clapham and Neer, 1997). All GPCRs have a conserved structure of seven transmembrane α-helices forming three extracellular loops, including an NH_2 terminus, and three intracellular loops, including a COOH terminus (Johnson, 2006). The third intracellular loop of the adrenergic receptor is the site of interaction with the guanine nucleotide-binding regulatory protein (G protein); activation of the adrenergic receptor causes a conformational change in the intracellular binding loop and subsequent dissociation of the G_{α} subunit (which is bound to GTP) from the $G_{\beta\gamma}$ dimer (Filipek et al., 2004).

There are at least nine different subtypes of adrenergic receptor, which are expressed throughout various body tissues (Table 3). Adrenoceptors can be activated by catecholamines either via neural synapses or via the circulation and are responsible for mediating a diverse array of physiological effects. Two major families of α-adrenoceptors have been identified: α_1- and α_2-, which are further subdivided into six subtypes α_{1A}-, α_{1B}- α_{1D}-, α_{2A}-, α_{2B}-, and α_{2C}-adrenoceptors. These play a role in regulating blood pressure, vasoconstriction (including regulation of blood flow through skeletal muscle) and cardiovascular performance. Numerous

secondary signalling pathways are activated by α-adrenoceptors, including phospholipases, Ca^{2+} channels, K^+ channels, Na^+/H^+ exchange pumps and the mitogen-activated protein kinase (MAPK) pathway (Saunders and Limbird, 1999).

The β-ARs are comprised of three main subtypes, β_1-, β_2-, and β_3-adrenoceptors. These are classically identified in cardiac, airway smooth muscle and adipose tissue, respectively (Johnson, 1998). The receptors consist of seven membrane-spanning domains, three intra- and three extracellular loops, one extracellular N-terminal domain, and one intracellular C-terminal tail (Johnson, 2006).

The most well-documented β-AR signalling pathway involves the cyclic AMP (cAMP)-protein kinase A (PKA) signalling pathway (Taskén and Aandahl, 2004a). In addition, PKA-independent cAMP signalling pathways are increasingly recognised, including the exchange protein directly activated by cAMP (Epac).

cAMP is involved in a huge number of biological processes and is able to control multiple processes within the same cell simultaneously. This is achieved through numerous mechanisms to ensure that the second messenger actions of cAMP are spatially and temporally regulated. These include:

1. Adenylyl Cyclase (AC): There are nine isoforms of AC, which are differentially expressed throughout body tissues. These isoforms differ in the way in which they respond to regulatory proteins, meaning that in a tissue or a cell type at a specific time, extracellular signals received through the adrenoceptors can be differentially integrated.
2. Phosphodiesterases (PDE): PDE are responsible for the degradation of cAMP, and therefore regulate the concentration of intracellular cAMP along with AC (Omori and Kotera, 2007). There are more than 50 different PDE proteins, encoded by 21 genes (Omori and Kotera, 2007). They differ in their tissue-expression, enzymatic activity, regulatory proteins and gene regulation.
3. PKA: PKA is composed of two regulatory and two catalytic subunits. When cAMP binds to two sites on each regulatory subunit a conformational change results in the release of the active catalytic subunit (Taskén and Aandahl, 2004b). These then phosphorylate various serine and threonine residues on proteins which initiate multiple signalling

pathways. In addition, the catalytic subunits can diffuse into the nucleus, where through the phosphorylation of the cAMP response element binding protein (CREB), they can regulate the expression of target genes (Mayr and Montminy, 2001).

4. Exchange proteins directly activated by cAMP (Epac): Epac are involved in a large number of cellular functions including cell division, adhesion differentiation and growth (Breckler et al., 2011). In addition, Epac proteins are expressed in distinct subcellular compartments such as the nucleus, the cytosol, nuclear and plasma membranes, allowing the compartmentalization of their signalling pathways (Breckler et al., 2011, p.; Gloerich and Bos, 2010).
5. A Kinase Anchoring Proteins (AKAP): Another group of proteins contributing to the compartmentalization and localization of cAMP-mediated signals are AKAP (Zaccolo and Pozzan, 2002). These are scaffolding proteins which bind to several cAMP messengers including PKA and Epac (McConnachie et al., 2006). AKAPs can also bind PDEs, creating temporal control over intracellular cAMP concentration (McConnachie et al., 2006).

An overview of β-AR signalling pathways is illustrated in Figure 10.

Adreno-ceptor	**Effector molecules**	**Expression**	**Main effect in muscle**	**Agonists**	**Antagonists**
$\alpha_{1A, B, D}$	PLC, PLA, PLD_2, Ca^{2+} channels	Coronary blood vessels, smooth muscle	Vasoconstriction	Phenylephrine	Prazosin, phentolamine
$\alpha_{2A, B, C}$	Inhibit cAMP, AC and PKA	Cerebral blood vessels, platelets	Vasoconstriction	Clonidine	Mirtazepine
β_1	Activate AC, increase cAMP and PKA	Heart and kidneys >> skeletal muscle	Unknown	Dobutamine, xamoterol	Metoprolol, bisoprolol
β_2	Increase cAMP, activate PKA, activate L-type Ca^{2+} channels	Heart, skeletal muscle	Hypertrophy	Salbutamol, formeterol, salmeterol, clenbuterol	Propranolol, ICI118,551
β_3	Increase cAMP, activate PKA, activate L-type Ca^{2+} channels	Heart, adipose tissue	Unknown	Mirabegron	Carvedilol

Table 3: Adrenoceptor subtypes, their expression and their effect in skeletal muscle.

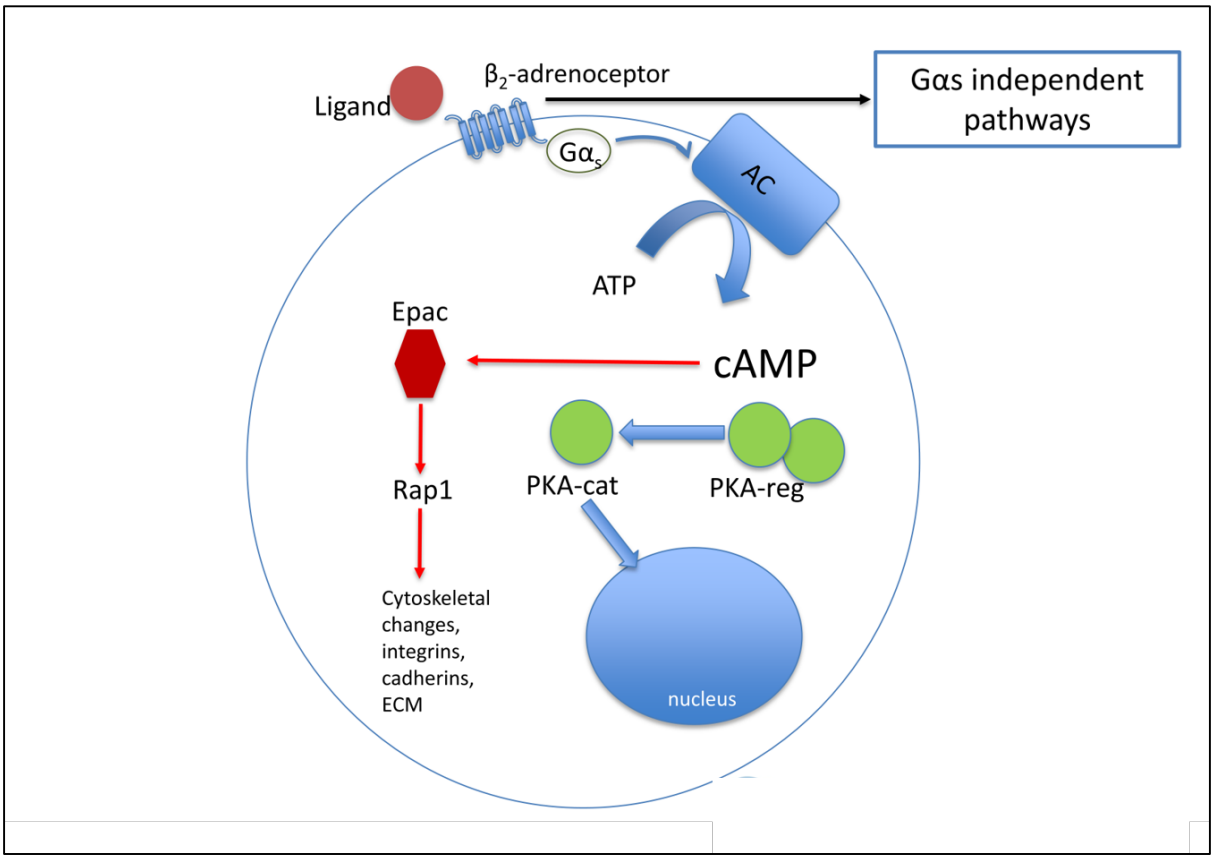

Figure 10: Schematic of β-adrenoceptor signalling pathways. β-Adrenoceptors belong to the G protein-coupled receptor family. Following β-adrenoceptor stimulation, the receptor couples to the Gαs subunit and activates adenylate cyclase, generating cAMP. A major effect of cAMP is the activation of cAMP-dependent protein kinase A (PKA), which then enters the cell nucleus and phosphorylates the ubiquitous transcription factor CREB. CREB increases the expression of cAMP-inducible genes containing the cAMP response element (CRE) sequence in mitochondria, myocytes, neurons, astrocytes, microglia and immunocytes. Other PKA catalytic subunits diffuse into the cytoplasm and nucleus and phosphorylate target proteins. In addition, increase in intracellular cAMP activates pathways via exchange protein activated directly by cAMP (Epac, also referred to as cAMP regulated guanine nucleotide exchange factors). Pathways activated by Epac are PKA independent. In addition, several pathways independent of Gαs have recently been discovered. ECM: extracellular matrix; PKA-cat: PKA catalytic subunits; PKA-reg: PKA regulatory subunits.

1.4.2 Adrenergic signalling in skeletal muscle

The importance of adrenergic signalling in skeletal muscle is now increasingly recognised, in particular for its role in skeletal muscle growth and repair (Lynch et al., 2007). The predominant adrenoceptor in skeletal muscle is the β_2AR, although β_1ARs comprise almost 10% (Williams et al., 1984).

Many β_2AR signalling pathways have been investigated in skeletal muscle. The AC-cAMP-PKA pathway is the most well characterized and is generally thought to be responsible for many of the changes in skeletal muscle growth which are mediated by the β_2AR (Lynch and Ryall, 2008).

The predominant isoforms of AC in skeletal muscle are AC2 and AC9, with small amounts of AC6 and AC7 (Suzuki et al., 1998). However, the specific roles of each isoform in skeletal muscle is not yet known (Lynch and Ryall, 2008). AC2 and AC9 have been found to be expressed at high levels in the neonatal mouse, suggesting a role in skeletal muscle development (Suzuki et al., 1998). β_2AR signalling has also been found to activate the Epac signalling pathway in skeletal muscle, and this accounts for some part of the β_2AR agonist induced hypertrophy of skeletal muscle (Shi et al., 2007). An abundance of Epac activity in fast-twitch muscle may also account for some of the fibre type-specific hypertrophy seen with β_2AR agonists (Shi et al., 2007). Skeletal muscle also contains multiple isoforms of AKAP. Muscle AKAP (mAKAP) has been identified at the sarcoplasmic reticulum (SR) indicating that it may play a role in the targeted phosphorylation of proteins and other receptors (e.g. the ryanodine receptor) in and around the SR (McCartney et al., 1995; Ruehr et al., 2003).

1.4.3 β-adrenoceptor agonists

1.4.3.1 Overview

One of the most important characteristics of adrenoceptors is that catecholamines can elicit a variety of responses depending on the adrenoceptor subtype bound. This means that

adrenoceptors can be targeted specifically by synthetic adrenoceptor agonists and antagonists (Table 3).

βAR agonists have been used to treat bronchoconstriction in asthma and chronic obstructive pulmonary disease for more than a century. These can be divided into two broad categories, short-acting β-agonists (SABA) and long-acting β-agonists (LABA). Given that it is β_2AR which is predominantly expressed in skeletal muscle, the rest of this summary focusses on β_2AR agonists.

The accepted model of agonists binding to the of the β_2AR is one in which the agonist is bound within the hydrophobic core of the protein and anchored there by specific molecular interactions between amino acid residues in the receptor and functional groups on the ligand (Johnson, 2006). The way in which a β_2AR agonist interacts with the β_2AR is determined by its molecular structure. Salbutamol, which is a SABA, is hydrophilic and therefore can access the active hydrophobic β_2AR binding site directly (Johnson, 2008). This means it will have a rapid onset of action (within 5 minutes) (van Noord et al., 1998). This structure also accounts for the short duration of action of salbutamol (4-6 hours) as the time that it binds to the active site of the receptor is limited (Johnson, 2008). LABAs, such as formoterol, are lipophilic, and therefore have to be taken up into the cell membrane before interacting with the receptor (Johnson, 2008). This means that the onset of action is slightly delayed, and the duration of activity is longer (Ringdal et al., 1998).

The potency of a drug (i.e. the amount required to produce a given effect) depends on affinity and efficacy. The affinity of a ligand is a measure of the strength of the molecular force binding it to the receptor. Salbutamol has a relatively low affinity for the β_2AR, compared to salmeterol, formoterol or isoprenaline (Johnson, 2006). Efficacy is the relationship between receptor occupancy and the ability to initiate a response. Most β_2AR agonists have intermediate efficacy and will behave as full agonists if receptor numbers permit. If receptor density or receptor coupling is inadequate, some β_2AR agonists can behave as partial agonists. Isoprenaline and formoterol are full agonists, whereas salbutamol and terbutaline are partial agonists (Johnson, 2008).

1.4.3.2 Side effects of β-adrenoceptor agonists

Many of the side effects of β_2AR agonists are pharmacologically predictable as a result of the adrenaline-like stimulation of the β_2AR. Tachycardia is common, particularly on first exposure to βAR agonists, and occurs due to a combination of peripheral vasodilation and subsequent reduced venous return and direct stimulation of the cardiac muscle (Lipworth et al., 1988; Sears, 2002). Cardiac arrhythmias are a much less common side effect, and tend to only occur in susceptible patients (Finn et al., 1997). Other studies have linked β-agonists with cardiac ischemia, heart failure, and cardiomyopathy, but these relationships are not confirmed (Sears, 2002). The LABAs salmeterol and formeterol have been shown to have greater effects on heart rate and on QTc interval than salbutamol (Bennett and Tattersfield, 1997; Malolepszy et al., 2001). In addition, studies in COPD patients with known cardiac arrhythmias suggest LABAs may be more cardiotoxic than SABAs (Cazzola et al., 1998). They may also decrease serum potassium more than SABAs which could lead to cardiac arrhythmia (Cazzola et al., 1998).

Tremor is a dose-related side effect of β_2AR agonists, which like tachycardia reduces with continued use. It is also more frequent with oral therapy than with inhaled therapy (Legge et al., 1971; Lipworth et al., 1988). The exact mechanism for tremor induction by β_2AR agonists is unknown. Early studies showed that β_2AR agonists reduced the force of tetanic contraction in slow muscles, and suggested that this was the primary mechanism leading to tremor (Waldeck, 1976). More recent studies have correlated tremor with reduced serum potassium (Lakie et al., 2004; Tesfamariam et al., 1998).

β_2AR agonists cause dose-dependent hypokalaemia by causing an intracellular shift of potassium (Bremner et al., 1993). In addition, through increasing glycogenolysis, β_2AR agonists can increase plasma glucose levels (Lipworth et al., 1988). As with tremor and tachycardia, these metabolic effects attenuate with repeated use (Lipworth et al., 1990).
In addition to these pharmacologically predictable side-effects, several studies have confirmed that frequent use of β_2AR agonists can cause airway hyperresponsiveness. This can occur after only short-term agonist exposure, and is thought to be due to tachyphylaxis of β-receptors on mast cells and a subsequent inflammatory response (Evans et al., 1997). This leads to a paradoxical increased asthmatic response, particularly induced by allergens and exercise (Kraan et al., 1985). This is particularly associated with fenoterol, salbutamol,

and terbutaline (Scalabrin et al., 1996; Wilding et al., 1996). Airway hyperresponsiveness is thought to account for unexpectedly high rates of morbidity and mortality in association with β_2AR agonist use during acute exacerbations of asthma (Sears, 2002).

1.4.3.3 β_2-adrenoceptor desensitisation

When β_2AR are stimulated by an agonist for some time, the response from the association of the receptor with the ligand is attenuated, a process known as desensitisation. Desensitisation can occur after short or prolonged agonist exposure and differs markedly between tissues. In short-term agonist exposure, the main mechanism underlying desensitisation is receptor phosphorylation. This is a transient process and can be quickly reversed on removal of the agonist. In prolonged agonist exposure, receptors are internalised resulting in a reduction in β_2AR density at the cell membrane. This can occur after hours of agonist exposure, and is also reversible within hours. Beyond this time period, β_2AR are downregulated due to ubiquitination of the receptors (Shenoy et al., 2001). Reversal of this requires an increase in βAR at the transcriptional level, and is therefore less easily reversible. Desensitisation of β_2AR is felt to account for the attenuation of clinical response seen after chronic agonist administration in heart failure and asthma (Billington et al., 2017; Bristow et al., 1982).

1.4.4 β_2-adrenoceptors and their agonists in Skeletal Muscle

There have been numerous studies on animals and several studies on humans regarding the effects of chronic β_2AR agonist administration on skeletal muscle. These were initially exploited by the livestock industry, but their potential for clinical use in muscle growth and repair was quickly recognised.

The most well recognised effect of β_2AR agonists on skeletal muscle is the repartitioning effect, i.e. an increase in skeletal muscle mass with a decrease in body fat (Yang and McElligott, 1989). In animal models these effects occur after 10-20 days of administration. The increase in muscle mass is cause by hypertrophy of individual muscle fibres and is due to a combination of an increase in muscle protein synthesis and reduction in protein degradation (Yang and McElligott, 1989). The hypertrophic effects occur in both fast and

slow twitch fibres, but there has been a lot of debate as to whether the magnitude of the effect is comparable in both fibre types (Lynch and Ryall, 2008). In addition to their anabolic effect, numerous studies have reported that β_2AR agonists can induce changes in fibre type, with a slow-to-fast fibre type switch (Agbenyega and Wareham, 1990; Hayes and Williams, 1994; Maltin et al., 1986; Zeman et al., 1987). Chronic clenbuterol administration in the mouse has been shown to alter myosin heavy chain (MHC) composition, however the mechanisms controlling this switch are not known (Lynch et al., 1996).

Because of these effects, the therapeutic potential of β_2AR agonists on skeletal muscle has been explored in several animal and human studies. Several studies in aged rats have shown that chronic administration of β_2AR agonists can increase muscle mass and force production (Ryall et al., 2007, 2006, 2004). In addition, several studies have shown that mouse models of muscular dystrophy, including the mdx mouse and the laminin deficient mouse, have improved muscle mass after β_2AR agonists administration (Agbenyega et al., 1995; Agbenyega and Wareham, 1990; Dupont-Versteegden, 1996; Dupont-Versteegden et al., 1995; Harcourt et al., 2007). Subsequent clinical trials focussed on the functional effects of salbutamol administration in muscular dystrophy. However, these trials have been limited in that equivalent doses to pre-clinical studies could not be achieved in humans due to cardiovascular side effects. In trials of fascioscapulohumeral muscular dystrophy, effects on muscle strength were very limited and treatment was limited by cardiotoxicity (Kissel et al., 2001, 1998). In a trial in Duchenne and Becker muscular dystrophy, a lower dose of salbutamol was well tolerated and increased lean body mass but had only modest effects on muscle strength (Skura et al., 2008). In addition, whilst β_2AR-agonist administration showed promising improvements in mouse models of ALS, again no improvement in muscle function was shown in clinical trials at tolerable doses in humans (Sorarù et al., 2006; Teng et al., 2006). Overall, whilst animal studies show clinical potential for β_2AR agonist administration for muscle wasting disorders, a greater understanding of βAR signalling in skeletal muscle is required if this pathway is to be manipulated for clinical use. Of particular necessity is the development of therapies that alter pathways benefitting skeletal muscle without simultaneously activating pathways that affect the cardiovascular system deleteriously.

1.4.5 β-adrenergic signalling at the NMJ

The innervation of skeletal muscle by sympathetic neurons was first established in the early 1900s, and subsequent studies sought to establish what role this innervation might play (Boeke J, 1909). In particular, several early studies provided evidence that symapthomimetics could alter neuromuscular transmission in preparations of isolated skeletal muscles in physiological salt solutions. These studies seemed to demonstrate wide-ranging and occasionally opposing effects of sympathomimetics depending on the context in which they were tested (Bowman and Raper, 1966). Adrenaline was shown to have a facilitatory action on neurotransmission in amphibian, mammalian and avian muscles following intra-arterial injection, where it lead to a rapid increase in CMAPs, and the amplitude of EPPs in the presence of the AChR antagonist tubocurarine (Bowman and Raper, 1967). This effect was re-produced to a lesser extent by noradrenaline, suggesting an α-adrenoceptor mediated effect. These effects were found to occur to an equal extent in both fast and slow twitch muscles, and were independent of vascular changes as evidenced by concomitant blood flow recordings (Bowman and Raper, 1966). In addition, adrenaline and noradrenaline can potentiate neuromuscular transmission, and activation of α_1 and β adrenoceptors has been shown to enhance nerve evoked ACh release (Burn, 1945; Vizi, 1991; Wessler and Anschütz, 1988). However, in conditions where neuromuscular transmission has already been blocked (by prior administration of tubocurarine) sympathomimetics demonstrate an opposing effect, leading to prolonged depth of paralysis (Bowman and Raper, 1966; Dybing, 1954). Many of these effects only occurred when sympathomimetics were used at higher concentrations than achieved in humans (Milone and Engel, 1996; Sieb and Engel, 1993).

Much of this early work was done in the belief that NMJs received a direct sympathetic innervation, and there exist several descriptions of an accessory innervation of skeletal muscle with endings of non-myelinated sympathetic fibres terminating within motor endplates (Hinsey, 1934). However, several subsequent studies demonstrated that these unmyelinated fibres were instead branches of motor nerves (Bowman and Nott, 1969; Bowman and Raper, 1967; Tiegs, 1953). Although the possibility was never entirely excluded, the accepted consensus became that the sympathetic effect on skeletal muscle

and possibly the NMJ was indirect on a humoral basis from the vascular sympathetic nerves, instead of involving sympathetic endings which act directly on the skeletal muscle fibre (Bowman and Raper, 1967).

Our ability to identify sympathetic nerves has greatly advanced since these early studies, which relied mainly on the diameter of the nerve and/or myelination to discriminate nerves of sympathetic origin. Sympathetic nerves can now be easily and specifically labelled with antibodies to tyrosine hydroxylase, neuropeptide Y or dopamine-β-hydroxylase (Kim et al., 2001; Yamamoto et al., 2000). Using this labelling, one recent study has shown evidence of sympathetic innervation of the NMJ in mice and enlargement of NMJ and upregulation of NMJ specific proteins in a mouse model of slow-channel CMS following β_2AR agonist (clenbuterol) administration (Khan et al., 2016). A subsequent study demonstrated that this innervation increases during postnatal development and is present in many muscle groups (Straka et al., 2018). This suggests a role for the sympathetic nerves at the motor end-plate in synaptic homeostasis.

The anatomical distribution of this possible sympathetic activity at the NMJ has clear relevance to the improvement in NMJ disorders seen with administration of exogenous sympathomimetics. The mechanisms by which sympathomimetics might mediate synapse integrity however, remain unknown.

Given the temporality of the observed effect of β_2AR-agonists in patients (a delayed and progressive improvement in weakness over months) it could be hypothesised that β_2AR agonists cause morphological restoration of the NMJ in certain subtypes of CMS. The pathology of Dok7 and ColQ CMS subtypes may be unified by the role of both proteins in post-synaptic differentiation and AChR clustering. Given that it is these subtypes in which the effect of β_2AR agonists is most established and dramatic, in particular β_2AR agonists may play a role in postsynaptic differentiation and maintenance. Indeed, several lines of evidence point towards cAMP/PKA dependent pathways involved in metabolic stabilization of AChRs (Khan et al., 2016; Poyner, 1992; Rudolf et al., 2013b). In addition, PKA is known to regulate the activity dependent morphological change that occurs to AChRs during early synapse development *in vivo* and *in vitro* (Nelson et al., 2003) (Li et al., 2001). Salbutamol has also been shown to enhance AChR clustering in C2C12 myotubes (Clausen et al., 2018).

1.5 Animal models of CMS

Animal models have been instrumental in understanding the role of many proteins in NMJ structure and function, and in investigating therapies which enhance neuromuscular transmission. Species used include rat and mouse, *C. elegans*, *drosophila*, frog, zebrafish, chick and canine models. The most frequently used, and arguably the most informative, has been the mouse model.

1.5.1 Mouse models of congenital myasthenic syndromes

The mouse NMJ is relatively large and easily accessible, and accurately reflects the human NMJ in many situations. While numerous differences between mouse and human NMJs exist, including morphological differences and temporal differences in development, many molecular pathways which impact on NMJ structure and function show considerable overlap (Tintignac et al., 2015). As previously discussed, knockouts of several NMJ proteins (agrin, rapsyn, MuSK, Dok7, laminins) lead to neonatal lethality. However, many CMS-related mouse models have been generated which exhibit myasthenic weakness and replicate the human disease to varying degrees (Table 4).

Experiments in this thesis utilised CollagenQ deficient mutant ($ColQ^{-/-}$) mice. Original founders of the $ColQ^{-/-}$ mouse line were provided by the Krejci laboratory, Inserm, Paris. This mouse line was obtained through homologous recombination. Male and female heterozygotes were mated to produce wild type and nullizygote littermates for the experiments in this thesis. $ColQ^{-/-}$ mice lack all asymmetric AChE at the NMJ. This is analogous with the ColQ CMS mutations in humans, which typically result in a complete absence of AChE at the NMJ (Ohno et al., 2000). The mice exhibit low body weight, approximately 50% of WT littermates, and limb muscle weakness (Feng et al., 1999). NMJs in $ColQ^{-/-}$ exhibit varying degrees of structural defects, with some synapses appearing smaller, immature or fragemented (Feng et al., 1999). However, these mice do survive into adulthood. This suggests that NMJs in these mice, as in ColQ CMS patients, have adaptive mechanisms to compensate for the lack of AChE.

Several objective assessments of muscle strength and fatigue can be used in characterising the phenotype and effect of treatment in these models. These include the inverted screen

test, grip strength test (forelimb or forelimb and hindlimb), and the treadmill fatigue test (Bonetto et al., 2015; Castro and Kuang, 2017). In neonatal mice, tests are more limited but the hindlimb suspension test and righting reflex can provide objective measures (Feather-Schussler and Ferguson, 2016). These tests are of principal importance, given the potential for poor correlation between abnormalities in NMJ morphology and NMJ function (Willadt et al., 2016).

Several important morphological differences between mouse and human NMJs have been characterised, and consideration of these is essential in interpreting pre-clinical studies using mouse models (Jones et al., 2017; Slater et al., 1992). Relative to the size of the muscle fibre, human NMJs are significantly smaller than mouse NMJs. In particular, the area of the motor nerve terminal and the area of AChR rich membrane are reduced (Slater et al., 1992). In addition, mouse NMJs are much less fragmented than humans (Jones et al., 2017). The extent of postjunctional folding in humans is much greater than in mice, meaning that despite reduced quantal content compared to mouse NMJs, neuromuscular transmission is significantly enhances by postsynaptic amplification (Slater et al., 1992).

In terms of the timeline development of the NMJ, very few studies exist in humans. In humans, synapse elimination occurs between 16 and 25 weeks in utero, compared to 2-3 weeks postnatally in mice (Brown et al., 1976; Hesselmans et al., 1993). In addition, NMJs change very little after the first year of life and remain very stable across the human lifespan (Hesselmans et al., 1993; Jones et al., 2017). This is in contrast to the apparent NMJ remodelling which takes place in mice (Jones et al., 2017; Valdez et al., 2012; Willadt et al., 2016).

CMS subtype	Gene	Protein	Allele type	Phenotype	NMJ abnormalities	Therapies tested
AChR deficiency(Missias et al., 1997)	*CHRNE*	AChR ε subunit	Knockout	Weakness apparent from 4w, lethality 10-12w.	Reduced AChR density, reduced AChR area	
Slow channel syndrome	*CHRNE, CHRND*	AChR ε subunit	Overexpression of mutant AChR εL269F or δS268F (Gomez et al., 1997)	Rapid respiratory rate, fatigable weakness from 6w, normal lifespan	Progressive myopathy and synaptic degeneration, focal Ca2+ deposition and accumulation of vacuoles	Fluoxetine(Zhu et al., 2015)
			Expression of εL221F mutation on *CHRNE* knockout (Chevessier et al., 2012)	Fatigable weakness from 6w, normal lifespan	Fragmented elongated endplates, damaged PJ folds	Ephedrine(Webster et al., 2013)
MuSK	*MUSK*	MuSK	V789M mutation hemizygous with MuSK knockout(Chevessier et al., 2008)	Reduced body weight, thoracolumbar kyphosis, weakness from P18, lethality 8-16w	Axonal overgrowth, loss of end-plate band, smaller and fragmented end-plates	
			Deletion of MuSK frizzled-like domain (MuSKΔCRD)(Messéant et al., 2015)	Fatigable weakness after 3w	AChR deficiency, motor axon outgrowth	Lithium chloride(Messéant et al., 2015)
Dok7	*DOK7*	Dok7	Homozygous for common c.1124_1127dupTGCC mutation (Arimura et al., 2014)	Severe weakness, low body weight, lethality within 2w	Small, immature NMJs	DOK7 AAV gene therapy (Arimura et al., 2014)
End-plate acetylcholinesterase deficiency	*COLQ*	ColQ	Knockout(Feng et al., 1999)	Weakness from P5, low body weight, 30% lethality at 12w, improvement with age	Variable NMJ morphology, muscle atrophy, reduced MuSK expression(Feng et al., 1999; Sigoillot et al., 2016, 2010)	
Agrin	*AGRN*	Agrin	Homozygous for p.Phe1061Ser mutation (*nmf380*)(Bogdanik and Burgess, 2011)	Myasthenic weakness from P13, atrophy, low body weight, lethality 12-16w	Progressive degeneration of NMJs, motor nerve sprouting and AChE loss	
GFPT1	*GFPT1*	Glutamine-fructose-6-phosphate transaminase 1	Muscle specific knockout(Issop et al., 2018)	Mild myasthenic weakness, normal lifespan	Reduced AChR area, reduced postjunctional folds, tubular aggregates in muscle	
VAMP1	VAMP1	Synaptobrevin	Spontaneously generated, homozygous null mutation(Nystuen et al., 2007)	Severe weakness and atrophy, lethality before 3w	Small NMJs, reduced EPP amplitudes	

Table 4: Summary of mouse models of congenital myasthenic syndrome

1.5.2 Zebrafish models of NMJ dysfunction

There are many factors which make zebrafish, *Danio rerio*, a useful model organism, including their ability to breed large numbers of offspring rapidly, their rapid *ex utero* development, their optical transparency and the ease of several methods of genetic manipulation which can be used (Gibbs et al., 2013). In addition, zebrafish offer several specific qualities making them an attractive model for the study of human neuromuscular diseases. Their motility and movement patterns can be easily and quantitatively measured in a reproducible way from just 24 hours post fertilisation (hpf). In addition, skeletal muscle is the largest organ of the developing zebrafish, and shares many structural and molecular features with mammalian muscles (Dou et al., 2008; Hirata et al., 2007; Parsons et al., 2002).

1.5.2.1 Muscle development in the zebrafish

Each muscle fibre type in zebrafish has distinct morphological and developmental properties (Figure 11) (Ochi and Westerfield, 2007). The first muscle fibres to differentiate are the slow twitch fibres. These migrate from a medial position near the notochord to the periphery of the somites by 16hpf. Following this, fast-twitch fibres differentiate and form the bulk of the somite (Bassett and Currie, 2003). By 24hpf, somitic muscle in the trunk of the zebrafish forms a series of pairs of chevron-shaped somites, divided by sheets of ECM, the vertical myosepta. Individual muscle fibres span the entire length of the somite, parallel to these vertical myosepta. The dorsal (upper) and lower (ventral) halves of each somite is further divided by another sheet of ECM, the horizontal myoseptum. The myoseptae (vertical and horizontal) are the main sites of NMJ formation (Blagden et al., 1997; Ochi and Westerfield, 2007).

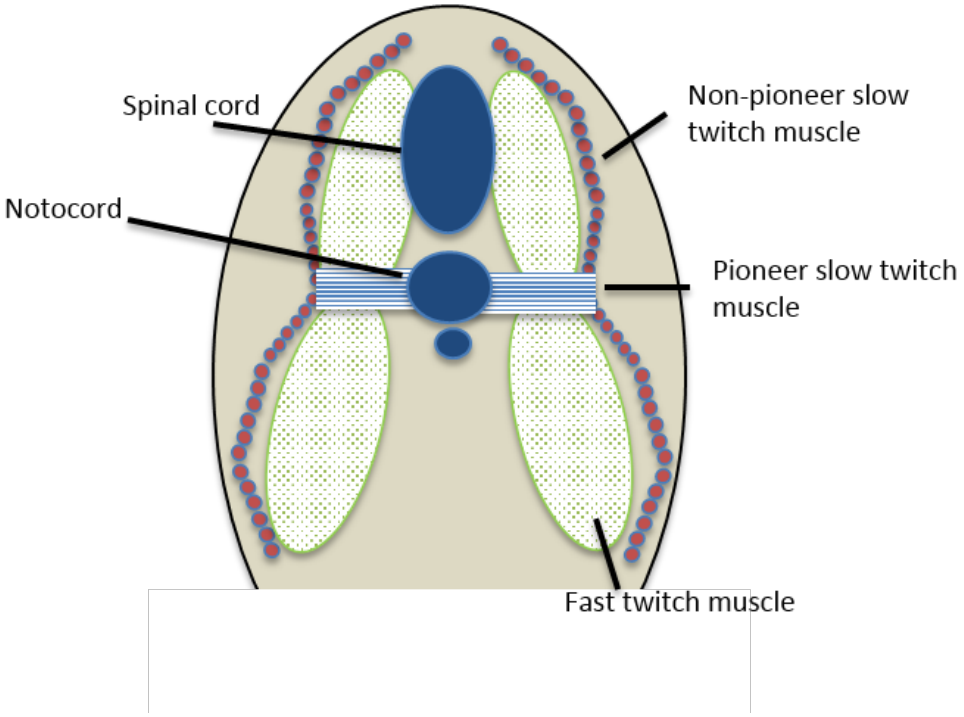

Figure 11: Schematic of cross-section of zebrafish muscle layers by 24hpf. Adaxial muscle cells migrate laterally to form a superficial monolayer of slow twitch muscle, whilst lateral presomitic cells remain deep and form a layer of fast twitch muscle

1.5.2.2 NMJ development in zebrafish

Zebrafish NMJs share many similarities with mammalian NMJs. Zebrafish NMJs are cholinergic and have pentameric AChRs. In addition, many of the key NMJ proteins in zebrafish have mammalian counterparts with identical or similar functions (Bayés et al., 2017).

In terms of NMJ development, this occurs in a similar stepwise manner in zebrafish as it does in mammals. Prior to arrival of the motor axons, a prepatterned band of AChRs form in the central region of adaxial muscle fibres, some of which will be incorporated into NMJs when the axonal growth cone makes contact between the nerve and muscle (Flanagan-Steet et al., 2005; Panzer et al., 2006). The first NMJs are formed between 16 and 24 hpf, and in parallel to the muscle somite development, the NMJs form in a rostral to caudal sequence (Myers et al., 1986; Westerfield et al., 1986). AChR clusters not incorporated into NMJs will disappear. By 120 hpf, most synapses are localized at the end of muscle fibres at

the vertical myosepta and are scattered over the entire length of muscle fibres (Westerfield et al., 1986).

1.5.2.3 Zebrafish models of congenital myasthenic syndromes

Studies on motor pathway choice in zebrafish have demonstrated that each segmentally arranged myotomal muscle of the zebrafish trunk is initially innervated by just three primary motor neurons, termed CaP, MiP and RoP, and each of these three primary motor neurons has a distinct spinal cord position and a stereotyped trajectory (Myers et al., 1986). Initially, all three motor growth cones extend along the medial surface of the somites toward a somitic "choice point" on the horizontal myoseptum (Myers et al., 1986; Schneider and Granato, 2003). Here, all growth cones pause and make contacts with muscle cells, and then select a cell-type specific path to ventral, dorsal or medial myotomal regions.

The speed at which the neuromuscular system develops, has allowed the consequences of mutations in key synaptic proteins that are lethal in mammals to be examined in zebrafish before death occurs, including functional knock-outs of AChRs, AChE, MuSK, rapsyn, GFPT1 and Dok7 (Müller et al., 2010a; Ono et al., 2001, 2002; Senderek et al., 2011; Westerfield et al., 1986; Zhang et al., 2004a). The ability to perform mutagenesis screens in zebrafish has revealed the molecular mechanisms underlying many aspects of NMJ development. The spontaneous zebrafish mutant phenotype *twitch once* is caused by rapsyn defects (Granato et al., 1996). This model lacks AChR clusters. In addition, rapsyn fails to localised to the postsynaptic membrane in these zebrafish (Ono et al., 2002). Other mutants include *nic1*, which lacks the α subunit of AChRs, *sofa potato*, which is due to a point mutation in the δ AChR subunit and lacks AChRs, *twister*, which has a gain-of-function mutation in the α AChR subunit and displays pre- and postsynaptic defects and the AChE mutants *ache* and *zieharmonika* which also show reduced AChR clustering (Behra et al., 2002; Downes and Granato, 2004; Ono et al., 2001; Westerfield et al., 1990). In addition, several point mutations of NMJ genes have been developed using random mutagenesis by use of *N*-ethyl-*N*-nitrosourea (ENU) (Daikoku et al., 2015). In many of these models, new functional roles for the synaptic proteins have been characterised.

zMuSK(*unplugged*) has two splice variants; SV1 which has a role in AChR prepatterning and axon guidance, and SV2 (full-length) which is dispensable for both of these processes (Jing et al., 2009; Zhang et al., 2004b). zMuSK transcripts are first detectable at 10 hpf and

expression is restricted to in adaxial muscle cells until late somitogenesis (>24hpf) when it is downregulated to coincide with the arrival of motor growth cones and lateral migration of adaxial cells (Zhang et al., 2004b). *Unplugged* null mutant zebrafish display axon guidance defects, with the motor axon stalling and/or producing abnormal branches at the choice point (Zhang et al., 2004b). The role of wnts in MuSK signalling was first identified in zebrafish, where it was shown that wnt11r binds to zMuSK, and that wnt11r, wnt4a and MuSK are mutually required for AChR prepattern (Gordon et al., 2012; Jing et al., 2009).

There are two Dok7 isoforms expressed in zebrafish. These are expressed from 10hpf when somatic segmentation begins, and peak at 19-24hpf, coinciding with NMJ formation. Functional knockdown using an antisense morpholino oligonucleotide was shown to lead to dramatic reduction in AChR prepattern, and reduced size of areas of synaptic contacts on the horizontal myoseptum (Müller et al., 2010b). In addition, these zebrafish morphants were shown to have structural abnormalities in slow muscle fibres, in-keeping with the myopathic features often found in Dok7 CMS patients (Müller et al., 2010b).

Zebrafish mutants lacking the MuSK and agrin binding domains of LRP4 have also been used to explore the specific roles of LRP4 in synapse formation (Remédio et al., 2016). Without LRP4, zebrafish fail to form en passant NMJs. However, although LRP4 is essential for AChR prepatterning in mice, it is not required for this process in zebrafish (Remédio et al., 2016).

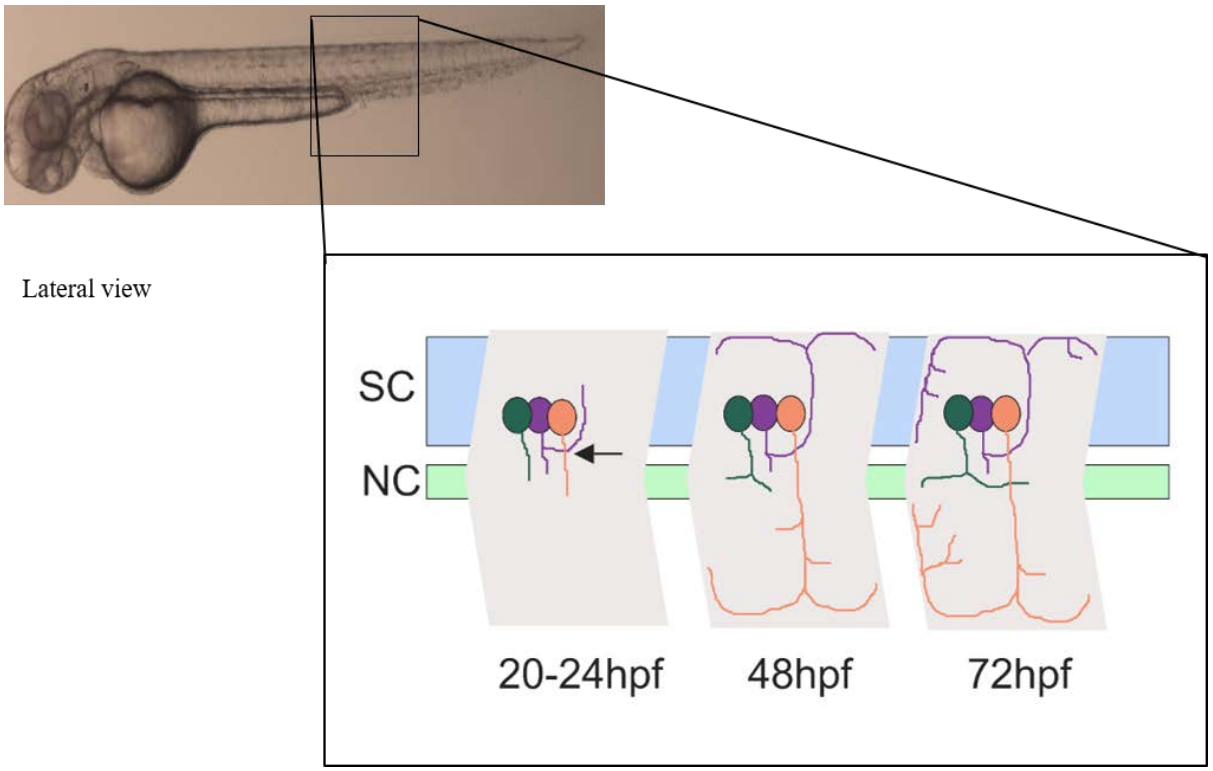

Figure 12: Schematic of the path taken by motor neurons in zebrafish somites during NMJ development. Primary CaP (orange), MiP (purple), and RoP (green) motor neurons project axons from the spinal cord. Each primary motoneuron projects its axon to a different area of muscle. Arrow marks the choice point on the horizontal myoseptum. SC: spinal cord. NC: notochord. Adapted from Panzer et al 2005.

Chapter 2. Thesis Objectives and Hypotheses

2.1 Thesis Objectives and Scope

The main aims of this thesis were

1. To improve our understanding of the role of β_2AR agonists in NMJ development by utilising zebrafish models of CMS
2. To explore the effect of β_2AR agonists in a CMS mouse model
3. To identify and delineate poorly understood aspects of the clinical features of CMS
4. To genetically characterise undiagnosed CMS patient cohorts

2.2 The beta-adrenergic agonist salbutamol modulates neuromuscular junction development in zebrafish models of human myasthenic syndromes

The mechanisms underlying the clinical benefit from β_2AR agonists in the treatment of CMS are not known. In this study, I utilised two zebrafish models to examine the effect of the selective β_2AR agonists on NMJ development. Many factors made these zebrafish models the ideal model to study the effect of salbutamol. The rapid development of the NMJ and large numbers of morphants tested meant that changes in NMJ morphology could be robustly quantified. This allowed me to confirm precisely how salbutamol altered NMJ morphology at each stage of NMJ development. In particular, the observed increased in AChR prepattern would not have been possible to observe in mammalian models. In addition, the use of zebrafish meant I could achieve functional knockdown of Dok7 and MuSK, something which would have been embryonically lethal in mouse models. I used morpholino oligonucleotides which inhibited pre-mRNA splicing of Dok7 and MuSK respectively, confirming reduction in mRNA levels by PCR. The effects of salbutamol on these models were compared to zebrafish injected with a control morpholino directed against human β-globulin mRNA. This study showed that salbutamol altered many steps of NMJ development in zebrafish with functional knockdown of Dok7 and MuSK, and could rescue motility defects in these models. In addition, using a selective β_2AR antagonist (ICI118,551) it was possible to block the effect of salbutamol, confirming that the mechanism of NMJ alteration was via the β_2AR, and not via an off-target effect. One final and exciting outcome of this study was that administration of forskolin replicated the

beneficial effects of salbutamol in these models. Forskolin is an AC activator, thus it directly increases cAMP without activating the β_2AR. This finding suggested that these patients may be amenable to alternative therapies which do not activate β_2ARs, which is of importance given the challenges of side-effects, cardiotoxicity and β_2AR desensitisation that arise with chronic β_2AR agonist use.

2.3 Salbutamol modifies the neuromuscular junction in a mouse model of ColQ myasthenic syndrome

Experiments in zebrafish pointed towards β_2AR agonists having a direct effect on the NMJ during development. However, this model was distant from the human condition in many ways. Apart from being a lower phylogenetic species, the use of the morpholino models meant that the effect of salbutamol could only be analysed at the embryonic stage of the zebrafish. In addition, it meant that salbutamol could only be administered for up to 5 days. In humans, the effect of salbutamol on muscle weakness is gradual, peaking after 6 months of treatment. In addition, in most CMS patients, it is not until clinical weakness develops and a diagnosis is made that treatment is started, which would usually be after NMJs are fully matured. I therefore sought to investigate the effect of salbutamol on the NMJ in a mouse model. I utilised the ColQ$^{-/-}$ mouse model, a model of AChE deficiency. AChE deficiency, along with Dok7, is a CMS subtype in which the beneficial effect of salbutamol is well established, and in which AChE inhibitors are often detrimental. This mouse model exhibits myasthenic weakness and NMJ abnormalities, but survives into adulthood. This allowed for a relatively long-term treatment regimen in these animals, with knockouts receiving 7 weeks of daily salbutamol via subcutaneous injections.

Expanding on the findings in the zebrafish models, 7 weeks of salbutamol lead to alterations in many parameters of NMJ structure in the ColQ$^{-/-}$ mouse. Predominantly, these changes were in the postsynaptic membrane. At an ultrastructural level, NMJs from mice treated with salbutamol had an increase in postjunctional folding, an effect which is likely to be functionally significant for neuromuscular transmission. Furthermore, mice treated with salbutamol had an increase in the expression of membrane bound MuSK, raising the possibility that salbutamol acts via a MuSK mediated pathway in this model.

2.4 Congenital myasthenic syndrome with episodic apnoea: clinical, neurophysiological and genetic features in the long-term follow-up of 19 patients

Congenital myasthenic syndrome with episodic apnoea (CMS-EA) is one of the least understood aspects of CMS. It was initially felt to be synonymous with mutations in *CHAT*, but several case reports and case series suggested it may occur in other genetic subtypes. CMS-EA is a cause of significant morbidity and mortality in children with CMS. In this study I sought to improve understanding of exacerbating factors, diagnostic clues, response to treatment and prognosis in patients with CMS-EA, in the hope of providing mechanistic insights into these events and of providing much needed clinical data on long-term outcomes. This was facilitated by the resource of tertiary referrals for clinical management and exome analysis for patients with CMS from around the world. From this cohort, 19 cases with CMS-EA were identified. The genotypes of these cases included mutations in *CHAT* but also *SLC5A7*, *COLQ*, *CHRNE*, *RAPSN* and *MYO9A*. This study revealed several unexpected findings. In particular, many of these CMS cases had CNS involvement, something that was not felt to be part of the CMS phenotype. Since the discovery of several recent presynaptic genes however, it is clear that many new CMS subtypes have a more complex presentation than a neuromuscular disorder alone. Several other findings pointed towards EA being a centrally mediated mechanism, although this remains to be confirmed. A relatively large proportion of CMS patients with this phenotype were undiagnosed when I identified them in 2017, suggesting the presence of additional presynaptic genes. In fact, since publication an additional three genes have been described which cause presynaptic defects and respiratory failure (*MUNC13-1*, *VAMP1*, *LAMA5*).

2.5 Impaired Presynaptic High-Affinity Choline Transporter Causes a Congenital Myasthenic Syndrome with Episodic Apnoea.

In two cases of CMS-EA undergoing exome analysis with collaborators at Inserm, Paris, mutations in the presynaptic high affinity choline transporter (encoded by *SLC5A7*) were identified. Mutations in SLC5A7 had not been associated with CMS before, although

dominant mutations are associated with distal hereditary motor neuronopathy (dHMN7). After reviewing clinical histories and examinations from the cohorts of genetically undiagnosed CMS referred to our centre, I identified 56 cases with phenotypic overlap with CMS due to *CHAT* mutations. After sequencing exons 1-10 of *SLC5A7* in these cases, two further cases carrying loss of function variants were identified. In the first case, two heteroallelic missense mutations were identified, c.524A>G (p.Tyr175Cys) and c.1030G>C (p.Val344Leu). The second case harboured two missense variants c.331T>C (p.Tyr111His) and c.1252T>G (p.Phe418Val). Segregation of the variants revealed that all had been inherited from heterozygous, healthy carriers apart from c.331T>C which had arisen de novo.

39 additional cases were sequenced by collaborators. In total, 4 cases with *SLC5A7* CMS were identified from a cohort of 95 genetically undiagnosed CMS-EA cases. Since publication, further cases of *SLC5A7* CMS have been described (Pardal-Fernández et al., 2018; Wang et al., 2017). Importantly, almost all cases show response to pyridostigmine, making the identification of this syndrome of great importance. The variants were confirmed to affect the activity of ChT in transfected HEK293T cells. In addition, the reduction of ChT activity was more severe in the variants associated with CMS-EA than in the previously reported variants associated with dHMN. This suggests that there may be a genotype-phenotype correlation in terms of the severity of the phenotype and the reduction in choline transport caused by the mutations.

Chapter 3.

3. The beta-adrenergic agonist salbutamol modulates neuromuscular junction development in zebrafish models of human myasthenic syndromes

McMacken G, Cox D, Roos A, Müller J, Whittaker R, Lochmüller H. The beta-adrenergic agonist salbutamol modulates neuromuscular junction formation in zebrafish models of human myasthenic syndromes. Hum Mol Genet. 2018 May 1;27(9):1556-1564.

Contribution: I designed and carried out the experiments. I carried out the morpholino injections, analysis of zebrafish motility, immunostaining and microscopy of whole mount zebrafish and image analysis using ImageJ. I analysed the results and wrote the main draft of the manuscript.

Chapter 4.

4. Salbutamol modifies the neuromuscular junction in a mouse model of ColQ myasthenic syndrome

McMacken G, Spendiff S, Whittaker RG, O'Connor E, Howarth RM, Boczonadi V, Horvath R, Slater CR, Lochmüller H. Salbutamol modifies the neuromuscular junction in a mouse model of ColQ myasthenic syndrome. *In Press*. Hum Mol Genet 2019

Contribution: I designed the salbutamol treatment protocol in the mouse models. I carried out analysis of muscle strength and body weight in the ColQ mice. I carried out immunostaining of whole mount muscle for NMJs and subsequent image analysis using ImageJ. I carried out sectioning and staining of gastrocnemius and soleus muscles and subsequent analysis of fibre type and area, and staining for additional NMJ proteins. I analysed the electron microscopy NMJ images using ImageJ. I drafted the manuscript.

Chapter 5.

5. Congenital myasthenic syndrome with episodic apnoea: clinical, neurophysiological and genetic features in the long-term follow-up of 19 patients

McMacken G, Whittaker RG, Evangelista T, Abicht A, Dusl M, Lochmüller H. Congenital myasthenic syndrome with episodic apnoea: clinical, neurophysiological and genetic features in the long-term follow-up of 19 patients. J Neurol. 2018 Jan;265(1):194-203

Contribution: I designed the study, collected the data and analysed the results. I drafted the manuscript.

Chapter 6.

6. Impaired Presynaptic High-Affinity Choline Transporter Causes a Congenital Myasthenic Syndrome with Episodic Apnoea.

Bauché S, O'Regan S, Azuma Y, Laffargue F, **McMacken G**, Sternberg D, Brochier G, Buon C, Bouzidi N, Topf A, Lacène E, Remerand G, Beaufrere AM, Pebrel-Richard C, Thevenon J, El Chehadeh-Djebbar S, Faivre L, Duffourd Y, Ricci F, Mongini T, Fiorillo C, Astrea G, Burloiu CM, Butoianu N, Sandu C, Servais L, Bonne G, Nelson I, Desguerre I, Nougues MC, Bœuf B, Romero N, Laporte J, Boland A, Lechner D, Deleuze JF, Fontaine B, Strochlic L, Lochmuller H, Eymard B, Mayer M, Nicole S. Impaired Presynaptic High-Affinity Choline Transporter Causes a Congenital Myasthenic Syndrome with Episodic Apnea. *Am J Hum Genet.* 2016 Sep 1;99(3):753-61.

Contribution: I identified 56 cases with a phenotype in-keeping with CMS-EA from a large cohort of possible CMS cases. I performed sequencing of exons 1-9 of *SLC5A7* in these patients. I identified pathogenic *SLC5A7* mutations. I clinically characterised these cases and contributed to the final draft of the manuscript.

Discussion and Future Work

The current treatment strategies for CMS are limited in that they are poorly understood, of varying efficacy and often limited by systemic side-effects. The optimum treatment regimen remains to be established and is difficult to study systematically given the individual rarity of these disorders. In many patients, optimum doses of sympathomimetics cannot be achieved due to side-effects and cardiotoxicity and despite promising pre-clinical studies in other neuromuscular disorders, widespread activation of β_2ARs limits clinical use. In order to develop more targeted therapies, an improved understanding of the mode of action of these drugs at the NMJ is essential.

Due to the known effects of β_2AR agonists on skeletal muscle (increased protein synthesis, reduced degradation, change in fibre type proportion), it has been hypothesised that the clinical benefit in CMS is primarily due to an anabolic effect in skeletal muscle. Indeed, the CMS subtypes which typically respond to these drugs are those which are also associated with myopathic changes on muscle biopsy (e.g. Dok7 and ColQ CMS). However, the findings in this thesis refute this by demonstrating that salbutamol directly affects NMJ structure in zebrafish and mouse models of CMS. Some findings pointed towards salbutamol altering MuSK dependent pathways, including the increase in prepatterning in zebrafish (a process which is orchestrated by MuSK) and an increase in the expression of membrane bound MuSK in the ColQ$^{-/-}$ mouse. Further studies will be required in order to determine whether MuSK activity is altered by salbutamol, and to explore which pathways upstream of MuSK are altered by β_2AR signalling.

What are the possible pathways by which β_2AR agonist induction of cAMP/PKA signalling could stabilize motor end-plates? The DGC stabilizes the myofibre membrane by linking the actin-based cytoskeleton to the basal lamina. The DGC also has well-evidenced roles in AChR clustering and AChE anchoring at the synaptic basal lamina (Bayne et al., 1984; Campanelli et al., 1994; Peng et al., 1999; Pilgram et al., 2010). Several components of the DGC physically associate with, or are phosphorylated by, PKA thus the activity of the DGC components could be modulated by the cAMP/PKA signalling pathway (Ceccarini et al., 2007; Fratini et al., 2012).

A further cell signalling pathway which is known to interact with the cAMP/PKA pathway is the Wnt signalling pathway. PKA inhibits the ubiquitination co-activator of Wnt, β-catenin, allowing its accumulation and the subsequent activation of the Wnt pathway (Hino et al., 2005). Another potential target is the ECM MuSK ligand biglycan, which has been shown to play key roles in synapse maintenance (Amenta et al., 2012). Biglycan regulates agrin induced MuSK phosphorylation and is particularly important in mature synapses, rather than during NMJ development (Amenta et al., 2012). Biglycan gene expression has been shown to be regulated by the cAMP/PKA pathway in other tissues (Ungefroren et al., 1998, 1997). In addition, a study using sympathetic ablation in mouse skeletal muscle demonstrated that the SNS may modulate levels of AChRs by regulating AChR turnover via the $G\alpha_{i2}$ -Hdac4-Myogenin-MuRF1pathway (A. C. Z. Rodrigues et al., 2019; Rudolf et al., 2013a). Thus multiple pathways could be activated by β-agonist cAMP/PKA signalling to induce AChR stabilization and modulation of key components of the post-synaptic apparatus.

Most evidence has pointed towards postsynaptic modulation by sympathomimetics. However, one recent study has focussed on the effect of β-agonists on presynaptic vesicle release (A. Z. C. Rodrigues et al., 2019). This showed that acute or chronic administration of salbutamol lead to an increase in synaptic vesicle release, and these effects could be blocked by reducing intracellular Ca^{2+}. This suggests that βAR agonists may have pre and postsynaptic effects (A. Z. C. Rodrigues et al., 2019).

My experiments in zebrafish showed that increasing intracellular cAMP downstream of the β_2AR could replicate the effects of salbutamol; in fact, the rescue effect of forskolin surpassed that observed with salbutamol. The diterpene derivative forskolin is an AC activator. Forskolin has been used in several clinical settings including asthma, congestive cardiomyopathy and obesity, and is safe and readily available. It is not currently in use in the treatment of CMS or any neuromuscular disorder, however. ACs are an important pharmacological target for many reasons. The nine AC isoforms have tissue-specific distribution, with isoforms 2, 6, 7 and 9 being expressed in skeletal muscle. In addition, as AC activation is receptor independent, it does not pose the same problems with loss of drug efficacy during long-term treatment. In follow up experiments, I am exploring how novel compounds which activate the muscle specific isoforms of AC can alter AChR clustering *in*

vitro. The development of a muscle specific AC activator could be an interesting therapeutic pathway in the management of CMS patients.

Despite huge advances in the last decade, many aspects of CMS are not understood. Episodic apnoea (EA) was one identified clinical feature on which data was lacking, and which is associated with significant mortality and morbidity. The study of a large cohort of CMS patients referred to our centre, revealed new insights into this condition, including prognostic factors, diagnostic pitfalls and genotype associations. This study highlights the value of data-sharing for rare diseases such as CMS. Without multi-centre collaboration, generation of clinical data on CMS-EA would be very limited.

The mechanisms underlying CMS-EA are yet to be defined, but understanding them will be essential in order to improve outcomes for this cohort. The cohort I identified had features which pointed towards a centrally mediated mechanism for recurrent apnoeic events. Respiratory rhythm is produced by two respiratory rhythm generators in the medulla oblongata, the parafacial respiratory group and the pre-Bötzinger complex (Koshiya et al., 2014; Smith et al., 1991). This respiratory rhythm is modulated by sites in the lower brainstem, including the Bötzinger complex, and in the pons, including the Kölliker–Fuse nucleus and the parabrachial complex (Alheid et al., 2004; Smith et al., 2007). Respiratory motor activities are then formed through premotor and motor-efferent networks including interneurons in the brainstem and spinal cord, which drive the muscles in the respiratory pump and the muscles regulating airway resistance (De Troyer et al., 2005). Cholinergic innervation and nAChRs are intrinsically involved in respiratory control in both the brainstem and spinal cord. Pontine cholinergic neurons can accelerate respiratory rate during REM sleep, and can activate breathing during wakefulness (Kubin and Fenik, 2004). AChRs can also modulate motor output of respiratory motor neurons (Bellingham and Funk, 2000). In addition, activation of AChRs in the pre-Bötzinger complex can modulate respiratory rhythm (Murakoshi et al., 1985; Shao and Feldman, 2001; Stewart and Anderson, 1968). Given the contribution of cholinergic signalling to respiratory control, it is possible that CMS mutations resulting in impaired ACh synthesis and release may disrupt the central neural control of respiration.

However, the discovery that *SLC5A7* mutations cause CMS-EA also raises an alternative explanation. The dHMN phenotype associated with *SLC5A7* mutations is associated with

vocal cord paralysis, raising the possibility that EA is caused by pharyngeal collapse. Another gene which is known to be associated with CMS-EA is *SCN4A* which encodes $Na_v1.4$ on the postsynaptic membrane. *SCN4A* mutations are more commonly associated with the skeletal muscle sodium channelopathies (hyperkalemic periodic paralysis, hypokalemic periodic paralysis, paramyotonia congenita and potassium-aggravated myotonia) which are caused by gain of function mutations (Lee et al., 2009). In infancy, these conditions are associated with brief, recurrent episodes of life-threatening respiratory muscle myotonia and laryngospasm causing apnoea, hypoxia, and cyanosis (Gay et al., 2008; Matthews et al., 2011; Yoshinaga et al., 2012). In addition, 4 cases with *SCN4A* mutations were recently identified from DNA of a cohort of 278 cases of sudden infant death syndrome (Männikkö et al., 2018).

In addition, examination of the cohort of CMS-EA cases revealed a relatively large proportion of these cases are genetically undiagnosed compared to CMS in general. Since the publication of this cohort, new presynaptic genes associated with CMS-EA have been identified. These patients have now all undergone whole exome sequencing, and analysis is ongoing for mutations in the newly described CMS genes and for novel presynaptic genes.

The identification of the novel presynaptic CMS gene *SLC5A7* is another treatable cause of CMS-EA associated with defects in ACh re-uptake and recycling. Dominantly and recessively acting *SLC5A7* mutations can lead to distinct clinical outcomes: a distal hereditary motor neuropathy with vocal cord paralysis (dHMN-VII) or a severe CMS-EA phenotype, respectively. Transmembrane *SLC5A7* mutations (causing CMS) lead to severely impaired CHT transporter function, which is reduced to a greater extent than the reduction caused by the cytoplasmic C-terminus mutations (associated with dHMN-VII). Nonetheless, the dHMN-VII associated mutations do exert a dominant-negative effect, as the reduction in choline transport is lower than would be expected in from haploinsufficiency alone (Barwick et al., 2012). This variable impact on choline transport activity goes some way to explain these two distinct phenotypes. In addition, cell surface expression studies indicate that transmembrane mutations may not be effectively transported to the NMJ, preventing any possible dominant negative effect (Wang et al., 2017). This provides a potential explanation for the lack of dHMN phenotype in heterozygous carrier parents of the *SLC5A7* CMS cases.

This finding is one of a series of recent discoveries highlighting the overlap between CMS and motor neuropathies. In an ongoing clinical study, I am further characterising this overlap using serial neurophysiological studies of the NMJ (RNS and SFEMG) in a cohort of patients with inherited motor neuropathies. In addition, this study will address the potential of therapeutic modulation of the NMJ with salbutamol in these conditions.

The introduction of next-generation sequencing strategies has accelerated the pace of rare disease gene discovery. A definitive molecular diagnosis can negate the need for further diagnostic investigations, facilitate appropriate access to treatment, provide prognostic information, facilitate genetic counselling and allow reproductive choices for families. In addition, understanding the genetic basis of these undiagnosed CMS subtypes will provide further information on the biological pathways at the human NMJ, and the development of targeted therapies. However, although substantial progress has been made, the underlying genetic basis for approximately 10% of cases remain undiscovered. These remaining unsolved cases are likely to be more complex for several reasons: the disease may have a lack of homogeneity of clinical phenotype, the disease may be ultra-rare, affected individuals may harbour variants which are not readily detected by whole exome/genome sequencing, and there may be a lack of clear investigative findings. The discovery of disease-gene associations requires confirmation of pathogenic variants in multiple unrelated individuals with an overlapping phenotype (Thompson et al., 2014). Therefore, data-sharing using standardised frameworks and with careful phenotypical characterisation will be crucial if the discovery of CMS causative genes is to continue at the current pace.

Conclusions

In recent decades there have been enormous advances in our understanding of the physiology and pathology of neuromuscular transmission, and the group of diseases associated with NMJ dysfunction is ever more clinically diverse. Emerging in parallel is the increasing implication of the NMJ in diseases beyond congenital and autoimmune myasthenia as a secondary pathological target.

In many of these conditions, the underlying pathomechanisms remain unclear. Treatments for the range of diseases affecting the NMJ are still limited. Furthermore, important unanswered questions remain about some of the most commonly used drugs. The essential task of developing improved treatments depends on enhancing our understanding of these underlying pathological mechanisms.

A primary aim of this thesis was to address the effect of βAR agonists in CMS. These experiments have demonstrated that salbutamol directly effects NMJ structure during development and maintenance, providing a possible explanation for the clinical benefit observed in patients. In addition, the data generated on the long-term outcomes of patients with CMS-EA will provide valuable information in a clinical setting, and insights into the mechanism underlying this unexplained clinical feature. Finally, through careful phenotyping of undiagnosed CMS patients, this work has contributed to the discovery of a novel presynaptic CMS gene.

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The beta-adrenergic agonist salbutamol modulates neuromuscular junction formation in zebrafish models of human myasthenic syndromes

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Abstract

Inherited defects of the neuromuscular junction (NMJ) comprise an increasingly diverse range of disorders, termed congenital myasthenic syndromes (CMS). Therapies acting on the sympathetic nervous system, including the selective β2 adrenergic agonist salbutamol and the α and β adrenergic agonist ephedrine, have become standard treatment for several types of CMS. However, the mechanism of the therapeutic effect of sympathomimetics in these disorders is not understood. Here, we examined the effect of salbutamol on NMJ development using zebrafish with deficiency of the key postsynaptic proteins Dok-7 and MuSK. Treatment with salbutamol reduced motility defects in zebrafish embryos and larvae. In addition, salbutamol lead to morphological improvement of postsynaptic acetycholine receptor (AChR) clustering and size of synaptic contacts in Dok-7-deficient zebrafish. In MuSK-deficient zebrafish, salbutamol treatment reduced motor axon pathfinding defects and partially restored the formation of aneural prepatterned AChRs. In addition, the effects of salbutamol treatment were prevented by pre-treatment with a selective β2 antagonist. Treatment with the cyclic adenosine monophosphate (cAMP) activator forskolin, replicated the effects of salbutamol treatment. These results suggest that sympathomimetics exert a direct effect on neuromuscular synaptogenesis and do so via β2 adrenoceptors and via a cAMP-dependent pathway.

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Introduction

The development of the neuromuscular junction (NMJ) requires several levels of organization, and occurs in a series of overlapping steps which require interplay between presynaptic nerves and postsynaptic muscle components. NMJs assemble in a narrow central region of the myofibre, where the density of acetylcholine receptors (AChRs) must be high in order to initiate a synaptic potential (1). Prior to innervation, in the step termed prepatterning, clusters of AChRs localize in a central band on muscle fibres (2). Several intrinsic muscle proteins are required for this process including the muscle-specific receptor tyrosine kinase (MuSK) and the low-density lipoprotein receptor-related protein-4 (LRP4) (3,4). Following innervation, some of these prepatterned AChR clusters are actively incorporated into developing NMJs, supporting the view that the postsynaptic apparatus defines its central region for ingrowing motor axons (5). The arriving motor nerve, and the agrin released by it, subsequently stabilize the AChR clusters and cause the dispersal of non-synaptic AChR clusters (6). Agrin binds the preformed MuSK/LRP4 complex to initiate MuSK autophosphorylation and activation, thereby initiating a cascade of signalling pathways leading to AChR clustering and postsynaptic differentiation (7,8). MuSK also recruits the adaptor protein Downstream of Kinase-7 (Dok-7), which stimulates further MuSK phosphorylation (9,10). Thus, the LRP4/MuSK/Dok-7 agrin receptor complex is indispensable for tyrosine phosphorylation and clustering of AChRs.

Components of the LRP4/MuSK/Dok-7 complex are the targets of mutations responsible for subsets of congenital myasthenic syndromes (CMS), which are characterized by fatigable muscle weakness (11). CMS are one of the few neuromuscular diseases for which symptomatic treatments are readily available. For many subtypes, clinical benefit is gained from administration of acetylcholinesterase (AChE) inhibitors, which augment the synaptic response to acetylcholine. For other CMS subtypes, including those with mutations that cause deficits in Dok-7, MuSK, and in end-plate AChE deficiency, the sympathomimetics ephedrine and salbutamol are first-line treatment. These drugs lead to increasing and sustained improvements in muscle strength, with the effect peaking after 3–6 months of commencing treatment (12–14). However, the mechanism by which these drugs may alter neuromuscular transmission in these patients is not known.

In order to address this, we examined the effects of the β2 agonist salbutamol on NMJ development in zebrafish. There are several factors which make zebrafish a useful model system to study synaptogenesis. NMJ development occurs in a similar series of steps in zebrafish as in mammals, with a diffuse elongated band of prepatterned AChRs forming in the central region of adaxial muscle fibres prior to the arrival of the motor growth cone (5). The first NMJs are formed between 16 and 24 h post fertilization (hpf) and by 120 hpf synapses are localized at the end of muscle fibres (the vertical myosepta) and scattered over the entire length of muscle fibres (15,16). The speed at which the neuromuscular system develops has allowed the consequences of deficiency of key synaptic proteins which would be lethal in mammals to be examined in zebrafish before death occurs, including functional knockouts of AChRs, AChE, MuSK, Rapsyn and Dok-7 (17–21).

Here, we use antisense morpholino oligonucleotides (MO) which knockdown expression of two key postsynaptic proteins, Dok-7, which leads to impaired motility and smaller NMJs, and *unplugged*, the zebrafish orthologue of MuSK, which demonstrates aberrant motor axon pathfinding and impaired AChR prepatterning (19,21,22). We show that salbutamol leads to improvement in AChR clustering, motor axon guidance and the development of prepatterned AChR clusters, as well as having functional benefit on motility and swim behaviour in zebrafish embryos. In addition, we show that the primary effect of salbutamol on NMJ development is mediated via β2 receptors and via the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway. Our results suggest that β2 agonists directly influence synaptic organization and that their therapeutic benefit in myasthenic disorders may be through morphological restoration of the NMJ.

Results

Salbutamol treatment of Dok-7 morphant zebrafish improves motility and synaptogenesis

In zebrafish, knockdown of NMJ proteins usually affects motility and swimming behaviour. The first locomotor behaviour stage observed is spontaneous alternating tail movements inside the chorion from 17 hpf. Dok-7 MO-injected embryos perform these at a reduced frequency to wild-type (WT) embryos, when measured at 24 hpf (21). Following 24 h of salbutamol treatment, Dok-7 embryos performed these tail twists at an increased frequency compared with untreated Dok-7 embryos (Fig. 1A, $P < 0.01$). After hatching from the chorion, zebrafish embryos respond to touch stimulus rapidly by swimming away from the stimulus. Approximately 50% of Dok-7 embryos demonstrate an abnormal response to touch stimuli, and may make abnormal twitching movements or fail to move away from the stimulus (21). Following salbutamol treatment, an increased percentage of Dok-7 embryos demonstrated normal swimming behaviour following touch stimuli (Fig. 1B, $P < 0.01$).

The morphology of the NMJ was examined in the Dok-7 zebrafish by immunostaining of AChRs and presynaptic nerve terminals (Fig. 2A). Although Dok-7 zebrafish lack prepatterned AChR clusters, they do subsequently develop AChR clusters opposed to the growth cone along the horizontal midline of each myotome (the horizontal myoseptum). In Dok-7 embryos, this area of pre- and postsynaptic co-localization is smaller than in WT embryos. We calculated the area of co-localization following salbutamol treatment as a percentage of the area of the myotome it occupied. Following salbutamol treatment, the area of co-localization on the horizontal myoseptum increased significantly (Fig. 2B, 6.2% in treated Dok-7 embryos, compared with 5.5% in untreated Dok-7 embryos, $P < 0.01$). In addition, the number of AChR clusters larger than 20 μm^2 on myotomal muscle fibres increased following salbutamol treatment (Fig. 2C, mean number of AChR clusters per myotome 2.9 in treated Dok-7 embryos, compared with 1.49 in untreated Dok-7 embryos, $P < 0.0001$).

Salbutamol treatment alleviates axon pathfinding defects in zMuSK zebrafish

The results of salbutamol treatment in the Dok-7 zebrafish prompted us to explore the effect of salbutamol on NMJ development when another key postsynaptic protein was knocked down. We studied the effect of salbutamol in *unplugged* morphants, the zebrafish homologue of MuSK. *Unplugged* has two splice variants; splice variant 1 (SV1) which has a role in AChR prepatterning and axon guidance, and splice variant 2 (full-length, FL) which is dispensable for both of these processes (19,22). We used a MO targeting only the *unplugged* SV1

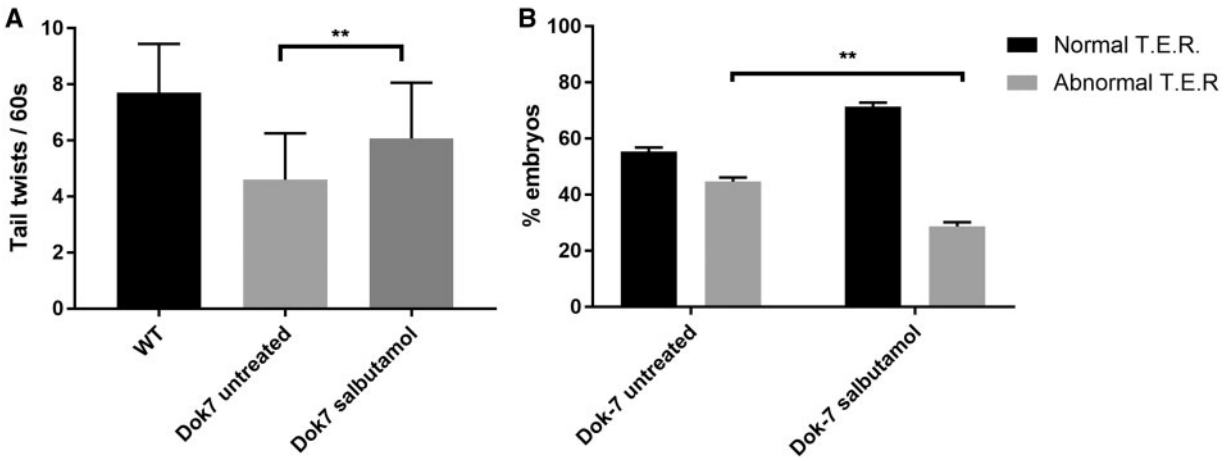

Figure 1. Dok7 morphant zebrafish demonstrate improved motility in the presence of salbutamol. (**A**) When monitored at 24 hpf, Dok7 MO-injected embryos are able to perform tail twisting movements in their chorion but do so at lower frequency than WT embryos. Following salbutamol treatment, Dok7 embryos performed tail twists at an increased frequency (mean 6.1 per 60 s) compared with untreated embryos (mean 4.6 per 60 s). A total of 30 embryos were observed for each category. ** indicates $P < 0.01$, Student's t-test, $n = 30$ treated and 30 untreated embyros observed. (**B**) Dok7 embryos demonstrate equal percentages of normal and abnormal touch-evoke response (TER) to stimuli at 48 hpf. Embryos treated with salbutamol demonstrate an increased percentage of normal swim behaviour. ** indicates $P < 0.01$, chi-square test, $n = 3$ tests on 30 embryos were performed for each category. All error bars depict S.E.M.

isoform (19). *Unplugged* SV1 transcripts are first detectable at 10 hpf and expression is restricted to adaxial muscle cells until late somitogenesis (>24 hpf) when it is downregulated to coincide with the arrival of motor growth cones and lateral migration of adaxial cells, thus embryos were examined in the first 24 hpf (19). We refer to zebrafish embryos with MO knockdown of *unplugged* SV1 as zMuSK.

In zebrafish, each myotomal muscle is initially innervated by just three primary motor neurons, termed CaP, MiP and RoP (16). Initially, all three motor growth cones extend along the medial surface toward a 'choice point' on the horizontal myoseptum (16,23). Here, growth cones pause and make contacts with muscle cells, and then select a specific path to ventral, dorsal or medial myotomal regions.

In contrast to WT embryos, zMuSK embryos display characteristic stalling and branching at the choice point, even though their dorsal adaxial cells are properly specified and migrate correctly (19). Following treatment with salbutamol, zMuSK MO-injected embryos displayed a restoration of axonal pathfinding defects at 24 hpf (Fig. 3A and B); in untreated embryos, 51% ($n = 123/240$) of imaged somites displayed axons which failed to cross the midline at 24 hpf, compared with 14% ($n = 34/240$) in embryos treated with salbutamol ($P < 0.0001$). In addition, zMuSK knockdown causes complete absence or greatly reduced numbers of AChRs at 24 hpf. In zMuSK MO embryos treated with salbutamol, however, AChR clusters could be seen as a band on the horizontal myotome at 24 hpf (Fig. 3C and D), which was similar although not as organized as the band seen in WT embryos.

In order to ascertain whether salbutamol treatment had an effect on the formation of prepatterned AChR clusters, we examined the caudal segments of embryos at 17 hpf for the presence of AChR clusters prior to the arrival of the motor growth cone. In salbutamol-treated embryos, a partial rescue of the formation of prepatterned AChR cluster formation was observed (Fig. 3E and F).

The effects of salbutamol on NMJ development are via the β2 receptor

In order to ascertain whether the effects seen following salbutamol treatment were due to its action as a β2 agonist, or via an off-target effect, we pre-treated zebrafish embryos with the selective β2 antagonist ICI118, 551. Following 3 h of pre-treatment with this β2 blocker, the addition of salbutamol failed to have the same effect on motor axon growth cones (Fig. 4), indicating that the effects observed are mediated via β2 receptors.

The effects of salbutamol on NMJ development are via the cAMP signalling pathway

Agonist activation of β2 adrenoceptors stimulates a cascade of intracellular signalling pathways, with the most prolific being the cAMP/PKA pathway. Following β2-adrenoceptor stimulation, the receptor couples to the Gαs subunit and activates adenylate cyclase, generating cAMP. However, several additional pathways which are Gαs-independent can also be activated by β2-adrenoceptors binding (24). In order to ascertain whether the downstream signalling pathway involved in rescue of the NMJ morphology in zebrafish was via cAMP or via a Gαs-independent pathway, we treated zebrafish embryos with the adenylyl cyclase activator forskolin. Following incubation for 24 h with 5μM forskolin, the effects of salbutamol axon pathfinding (Fig. 5A and B) and AChR clustering (Fig. 5C) in zMuSK embryos were replicated. In addition, forskolin treatment lead to an increase in the number of prepatterned AChR clusters (mean 3.5 prepatterned clusters per myotome in forskolin-treated embryos, compared with 0.5 in untreated embryos, $P < 0.0001$).

Discussion

The process of neuromuscular transmission is complex and involves several levels of specialization. There are numerous cellular and molecular pathways which may be potential pathological or therapeutic targets. Given that β_2 adrenoceptors activate many signalling pathways linked to a variety of changes in different tissues and cell types, the mechanisms of improved neurotransmission by β-agonists may be numerous, and may include postsynaptic expansion of end plates, growth of presynaptic nerve terminals or restoration of normal levels of neurotransmitter release. Zebrafish models allow the analysis of the effect of β-agonists during each step of NMJ development,

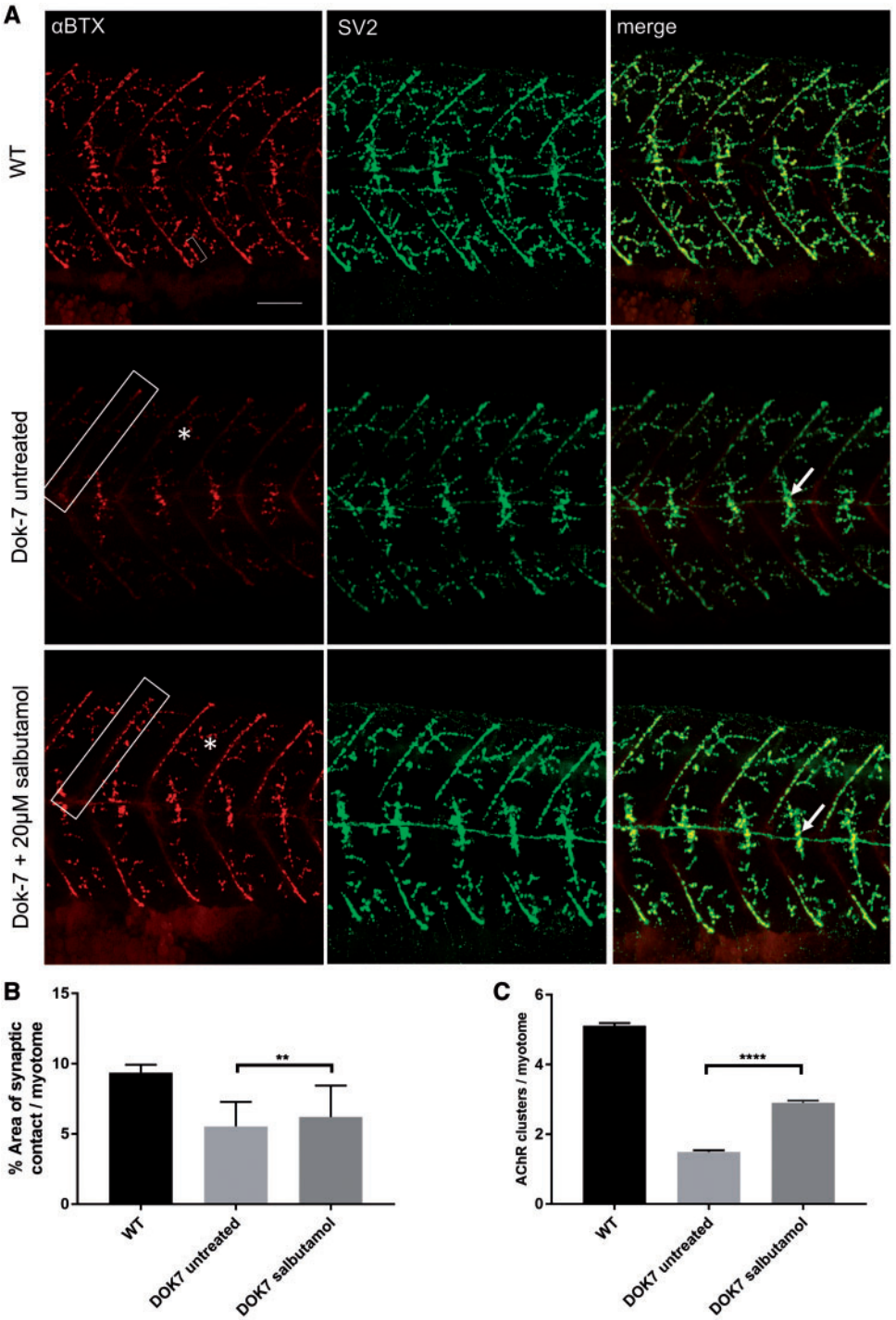

Figure 2. Salbutamol treatment of Dok-7 MO-injected zebrafish improves NMJ morphology. Lateral views of 48 hpf embryos with neuromuscular synapses labelled with antibodies against SV2 (green, presynaptic vesicles) and αBTX (red, postsynaptic AChRs). Scale bar = 50 μm. (**A**) In Dok7 MO-injected embryos, the focal innervation point along the horizontal myoseptum is reduced in size compared with wild type, but is increased following treatment with 20 μM salbutamol (arrows). In addition, the morphology of myoseptal (boxes) and myotomal (asterisks) AChR clusters is partially rescued with salbutamol treatment. A representative 20 μm^2 AChR cluster is labelled with a bracket. (**B**) Following salbutamol treatment, the area of synaptic contact on the horizontal myoseptum is significantly increased (** indicates $P < 0.01$, Student's *t*-test, $n = 160$ treated and 160 untreated myotomal segments examined). (**C**) Salbutamol treatment caused a significant increase in the number of AChR clusters >20 μm^2 per myotome in Dok7 zebrafish embryos (**** indicates $P < 0.0001$, Student's *t*-test, $n = 100$ treated and 100 untreated myotomal segments examined).

which in turn may give some indication as to which molecular mechanisms may be involved, based on the previously characterized role of key NMJ proteins at each of these steps. We observed that salbutamol treatment led to morphological improvement of the NMJ at several developmental steps: an increase in the area of co-localization of presynaptic nerve terminals and AChR clusters on the horizontal myoseptum in Dok-7 MO-injected embryos, improvement in AChR clustering at in Dok-7 MO-injected embryos, rescue of the axonal pathfinding defects in zMuSK MO-injected embryos and improvement in

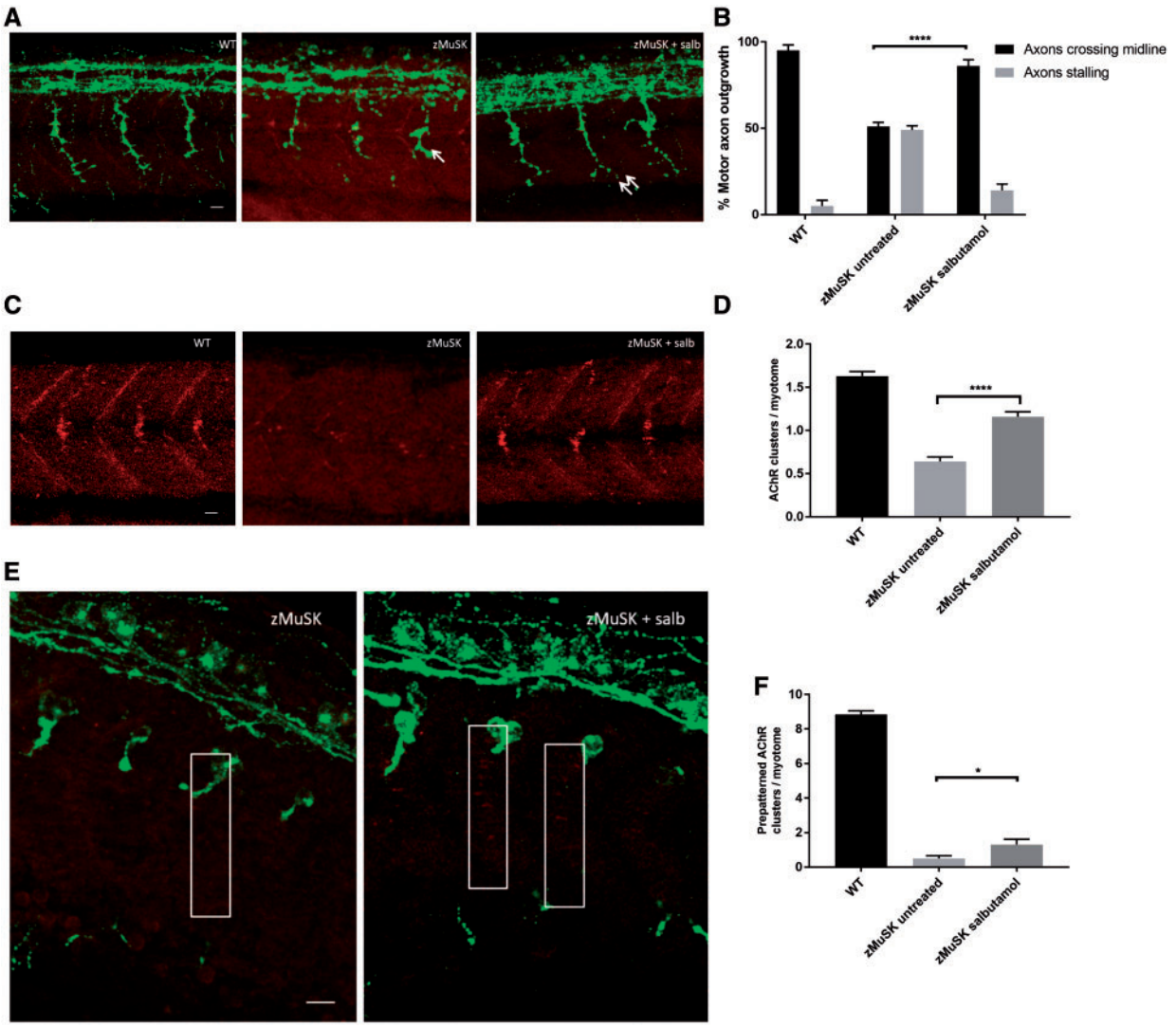

Figure 3. Salbutamol treatment of zMuSK MO-injected embryos improves motor axon pathfinding defects and AChR clustering. Lateral views of embryos with neuromuscular synapses labelled with antibodies against SV2 (green, presynaptic vesicles) and αBTX (red, postsynaptic AChRs). Scale bars = 10 μm. (**A**) zMuSK MO-injected embryos at 24 hpf demonstrate characteristic stalling of the outgrowing motor axon at the choice point on the horizontal midline (arrow). Following salbutamol treatment more axons cross the horizontal midline and extend to the periphery of the myotome (double arrow). (**B**) Quantification of the improvement of motor axon growth following salbutamol treatment. (**** indicates $P < 0.0001$, chi-square test, $n = 240$ treated and 240 untreated myotomal segments examined). (**C**) zMuSK embryos at 24 hpf display almost complete absence of AChR clustering. In salbutamol-treated zMuSK embryos, AChR clustering is restored. (**D**) Quantification of the improvement in AChR clustering following salbutamol treatment of zMuSK embryos. AChR clustering is significantly increased with the mean number of AChR clusters >20 μm^2 per myotome 1.16 in salbutamol treated embryos compared with 0.64 in untreated embryos (**** indicates $P < 0.0001$, Student's *t*-test, $n = 100$ treated and 100 untreated myotomal segments examined). (**E**) zMuSK MO-injected embryos at 17 hpf. Untreated embryos lack elongated, diffuse prepatterned clusters on the myotomal surface prior to the arrival of the motor growth cone (boxed areas), but following salbutamol treatment some zMuSK embryos display prepatterned clusters. (**F**) Quantification of the effect of salbutamol on prepatterned AChR clustering in zMuSK embryos. Following salbutamol treatment, the number of prepatterned AChR clusters >3 μm^2 in zMuSK embryos increased, with a mean of 0.5 prepatterned clusters per myotome in untreated embryos, compared with 1.3 in salbutamol-treated embryos (* indicates $P < 0.05$, Student's *t*-test, $n = 30$ untreated and 30 untreated myotomal segments examined).

the formation of prepatterned AChR clusters. We did not observe an improvement in the number of prepatterned AChR in Dok-7-deficient zebrafish (data not shown). This is unexpected, given the known function of the MuSK/Dok-7 complex at the NMJ, and it is possible that changes in expression of prepatterned AChR in the Dok-7 MO-injected embryos may have been too small to be detected as changes on immunostaining. In addition, we observed that the adenylyl cyclase activator forskolin, lead to dramatic improvements in axonal pathfinding and AChR clustering, and improved AChR prepatterning in zMuSK zebrafish.

Each of these steps are governed primarily by muscle-derived signals in zebrafish (19,25). This is in-keeping with the fact that it is mainly postsynaptic CMS subtypes in which the effect of β-agonist therapy is beneficial. Salbutamol treatment leads to an increasing and sustained response in Dok-7 CMS (13). Although the pathomechanisms of Dok-7 CMS are not yet fully understood, it is widely accepted that mutations in *DOK7* impair Dok-7's ability to activate MuSK. This assumption is supported by the fact that many mutations in Dok-7 CMS target the COOH terminal, phosphotyrosine binding (PTB) or pleckstrin homology (PH) domains, which are critical sites for activation of MuSK *in*

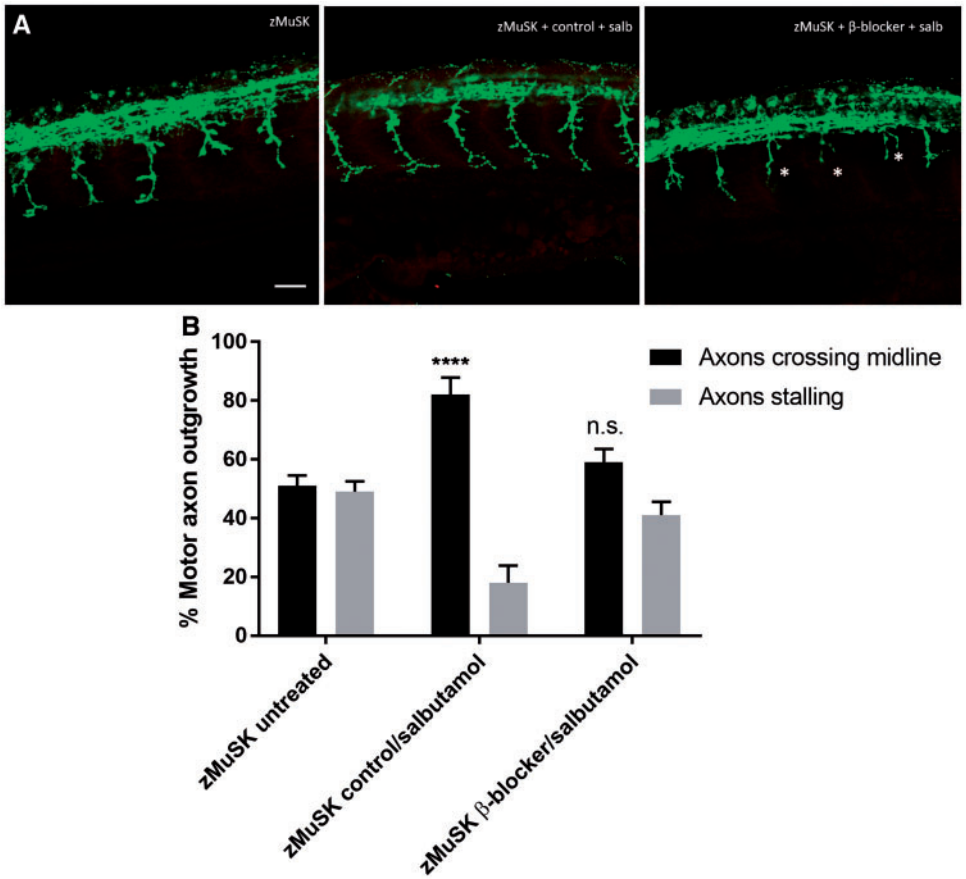

Figure 4. Pre-treatment with a selective β2 antagonist blocks the rescue of NMJ development by salbutamol. Lateral views of 24 hpf embryos with neuromuscular synapses labelled with antibodies against SV2 (green, presynaptic vesicles) and αBTX (red, postsynaptic AChRs). Scale bar = 50 μm. zMuSK embryos were treated with 5 μM of the selective β2 antagonist ICI118, 551 or control (water) added to E3 medium for 3 h prior to the addition of 20 μM salbutamol. (**A**) In zebrafish embryos pre-treated with ICI118, 551 no rescue of the axonal pathfinding defects was seen following 21 h of salbutamol treatment, with stalling and branching of motor growth cones (asterisks). (**B**) Quantification of the effect of pre-treatment of ICI118, 551 on motor axon growth. Following pre-treatment with 5 μM ICI118, 551, salbutamol treatment did not have an effect on axon pathfinding (n.s. $P > 0.05$, ****$P < 0.0001$, chi-square test, $n = 240$ treated and 240 untreated myotomal segments examined).

vitro and *in vivo* (26,27). The other CMS subtype in which treatment with β agonists leads to consistent improvement is CMS due to end-plate AChE deficiency, which is caused by mutations in *COLQ* (28,29). In addition to its role in anchoring AChE in the basal lamina, the C-terminus domain of ColQ binds MuSK, and ColQ has been shown to have an important regulatory role in postsynaptic differentiation through this interaction (30). The pathology of Dok-7 and ColQ CMS subtypes may be unified by the role of both proteins in postsynaptic specialization and AChR clustering. β agonist therapy may therefore also play a role in postsynaptic differentiation in mammals. Indeed, several lines of evidence point toward cAMP/PKA-dependent pathways involved in metabolic stabilization of AChRs (31–33).

The effect of salbutamol on both axonal pathfinding and number of prepatterned AChR clusters in zebrafish indicates a possible role for β2 agonists in upregulation or activation of a MuSK-dependent pathway in early synapse development, which has been previously shown to be indispensable for normal regulation of both of these processes in zebrafish (19). There are several potential pathways by which β2-adrenergic agonist-mediated activation of the cAMP/PKA pathway could be activating MuSK, including the β-catenin/Wnt signalling pathway. The zebrafish homologue of MuSK has been previously shown to interact with Wnt11r to restrict AChR prepattern in a central muscle zone (22), in a similar manner to the interaction with Wnt signalling and mammalian MuSK (34). However, given that salbutamol and forskolin treatment led to only partial rescue of the motility and NMJ defects in our zebrafish models, it is conceivable that additional pathways not upregulated by cAMP signalling are also involved.

The effects of forskolin on the NMJ not only indicate the cAMP mimetic action of salbutamol at the NMJ, but also were even more marked than those observed following salbutamol exposure. This more obvious effect may reflect increased absorption, bioavailability or efficacy of forskolin compared with salbutamol in zebrafish. Forskolin has been used in several clinical settings including asthma, congestive cardiomyopathy and obesity (35–37). However, its efficacy in neuromuscular disease is unknown. One possible role for forskolin could be in the treatment of CMS patients who have become tolerant to the effect of beta-adrenergic receptor stimulation following many years of treatment, given that its mechanism of action is receptor independent. Further preclinical studies will be required to confirm these findings.

We accept there are limitations to our disease models. Whilst treatment of zebrafish embryos provides a model for NMJ formation and exploring the role of β2 agonists in this process, effects can only be studied over a short treatment period. The therapeutic effect of β agonists seen in CMS patients is a delayed one, with an increasingly positive response after many months of treatment (13). The relatively short exposure duration of the zebrafish

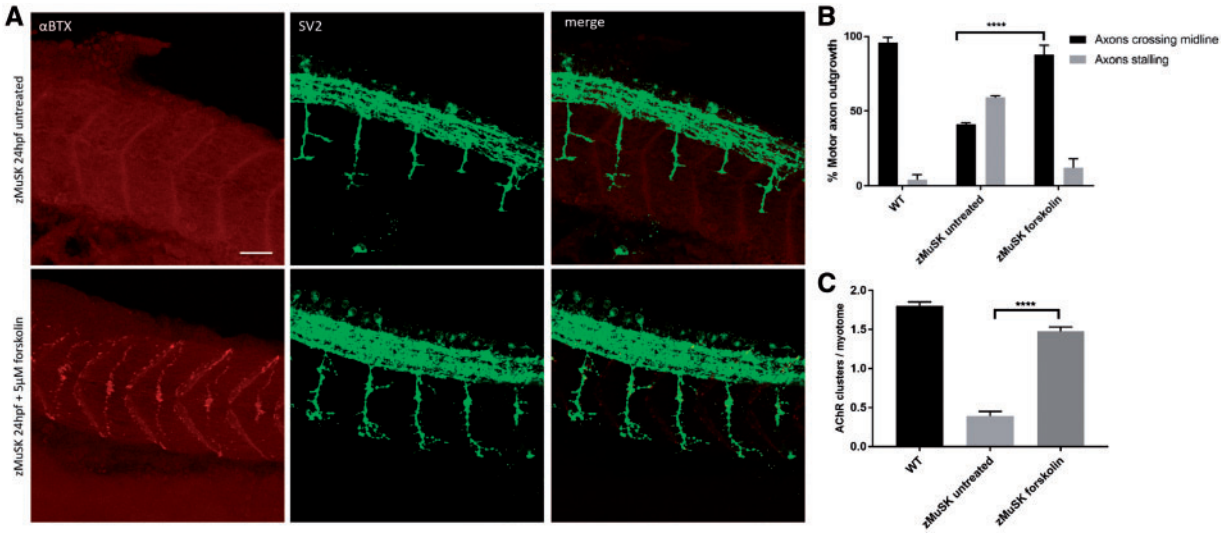

Figure 5. The effects of β2 receptor agonist activation on NMJ morphology are replicated by cAMP activation. Lateral views of 24 hpf embryos with neuromuscular synapses labelled with antibodies against SV2 (green, presynaptic vesicles) and αBTX (red, postsynaptic AChRs). Scale bar = 50 μm. (**A**) zMuSK embryos at 24 hpf demonstrate improvement in AChR clustering and axon pathfinding defects following forskolin treatment. (**B**) Quantification of motor axon guidance defects. About 88% ($n = 28/240$) of imaged somites of zMuSK embryos treated with forskolin displayed no axon guidance defects, compared with 41% ($n = 142/240$) of those in untreated zMuSK embryos. **** indicates $P < 0.0001$, chi-square test. (**C**) The number of AChR clusters larger than 20 μm^2 on myotomal muscle fibres increased with forskolin treatment, with mean number of clusters 0.39 per myotome in untreated embryos, and 1.48 in treated zMuSK embryos. **** indicates $P < 0.0001$, Student's *t*-test, $n = 100$ treated and 100 untreated myotomes examined.

to salbutamol and forskolin may further explain why only a partial rescue of the phenotype was observed in both models. In addition, patients with CMS due to *DOK7* mutations frequently present after infancy or in early adulthood (38), and postnatal knockdown of Dok-7 gene expression has been shown to cause CMS in mice (39), indicating a further role for Dok-7-mediated activation of MuSK in postnatal NMJ maintenance. Therefore, it is possible that the improvement seen in β-agonist-treated CMS patients is due to an additional role they play in NMJ homeostasis. This hypothesis is supported by the recent finding of sympathetic innervation in close contact with the NMJ contributing toward NMJ maintenance (32). Further work will be in identifying the mechanism of altered postsynaptic specialization by either endogenous or exogenous β2-adrenoceptor ligands and cAMP activation. Nevertheless, the effects demonstrated during NMJ development in these zebrafish models, indicate that sympathomimetics may alter the regulation of key NMJ signalling molecules, and suggest that β2 agonists illicit their therapeutic effect in myasthenic disorders through a direct effect at the NMJ.

Materials and Methods

Zebrafish husbandry

The Golden strain (slc24a5$^{b1/+}$) of zebrafish was used (Zebrafish International Resource Centre, Oregon). Zebrafish embryos were raised in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 0.01% methylene blue) at 28.5°C and staged in hours post fertilization (hpf) according to standard protocols.

Phenotype and motility observation

Tail twists in chorion at 24 hpf were measured by observing each embryo for 60 s and counting total number of complete twists. A total 30 embryos were measured for each category. Touch-evoked swimming response (TER) was observed by touching the head or tail of the zebrafish with a fine pipette tip. TER was defined as abnormal if embryos exhibited either circling movements, twitching with no movement away from the stimulus, or had no movement at all as a response. At least 20 embryos were observed for each category and the experiment was repeated five times.

Antisense morpholino oligonucleotide knockdown

Antisense MO were injected into the yolk of one-to-two cell stage embryos. Dok-7 embryos were injected with 15 ng of Dok-7 MO. MuSK embryos were injected with 5 ng of zMuSK MO. In addition, control MOs were injected at the same concentration for each injection experiment. MOs were purchased from Gene Tools LLC (Pilomath, OR). A splice-blocking MO was used to target Dok-7 transcripts at exon 2: 5′-ATTTATAGGATTTACCTG CTACCGG. This splice-blocking MO causes skipping of the exon and premature translation termination through targeting of the splice donor site of exon 2 (21). For MuSK experiments, a MO targeting the unplugged splice transcript variant 1 (unplugged/SV1) was used (19,22): 5′-GTAGAGGATTACCGTATTGCCGTT. This causes skipping of exon 2, frameshift and a premature stop codon after 24 cryptic residues. The Gene Tools standard control MO (5′-CCTCTTACCTCAGTTACAATTTATA-3′) targeting a human beta-haemoglobin gene was used as a negative control. MOs were suspended in 1× Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM $Ca(NO_3)_2$, 5 mM HEPES; pH 7.6) with phenol red as an injection indicator. At least two independent MO injection experiments were performed for each MO and 200–800 injected embryos were evaluated for each treatment regimen.

Treatment of zebrafish with salbutamol, ICI118, 551 and forskolin

Zebrafish embryos were raised post injection in E3 medium containing the compounds or control vehicle. We conducted

preliminary dose response testing for salbutamol (1, 10, 20 and 50 μM), forskolin (0.1, 1, 3, 5 and 10 μM) and ICI118, 551 (1, 3, 5, 20 and 50 μM). Concentrations of 20 μM salbutamol, 5 μM forskolin and 5 μM ICI118, 551 resulted in strong effects and limited toxicity, and these concentrations were selected for all further experiments. For salbutamol treatment, embryos were exposed to 20 μM concentration of salbutamol (Sigma) or 20 μM of methanol for up to 72 hpf. For pre-treatment with the selective β2 receptor blocker ICI118, 551, zebrafish embryos were incubated with E3 medium containing either 5 μM of ICI118, 551 hydrochloride (abcam) or 5 μM of distilled water. After 3 h, 20 μM of salbutamol was added to the same E3 medium. For forskolin treatment, zebrafish embryos were raised in E3 medium containing 5 μM of forskolin (abcam) or 5 μM ethanol for up to 72 hpf.

Zebrafish whole-mount immunofluorescence staining

Embryos were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) overnight and then permeabilized in cold acetone at −20°C. Depending on their age, 3–5 days post fertilization old larvae were permeabilized with collagenase A (Roche Diagnostics, 1 mg/ml) for 60 min. Embryos were blocked in 5% horse serum in PBS 0.1% Tween-20 (PBS-T). Embryos were incubated in 5% horse serum in PBS-T containing primary antibody overnight at 4°C (presynaptic nerve terminals: SV2, 1: 200 from Developmental Studies Hybridoma Bank, Iowa), washed several times with PBS-T and incubated with secondary antibody (goat anti-mouse Alexa Fluor 488, donkey anti-mouse Alexa Fluor 594, goat ant-rabbit Alexa Fluor 680, all Invitrogen). AChRs were visualized by using Alexa Fluor 594-conjugated α-bungarotoxin (1 μg/ml, Invitrogen). Immunofluorescence staining was imaged using a Nikon confocal microscope (Nikon A1R Invert). For quantification of NMJ morphological changes, 30 embryos were imaged for each category. Because of the rostral-to-caudal gradient of embryonic development in zebrafish, we used the same myotomal segments for comparison, by examining the five myotomes centred around the caudal-most part of the yolk extension. WT, untreated morphant and treated morphant tissues were processed for immunofluorescence in parallel and images were acquired with the same confocal microscopy settings (laser power, gain, magnification and Z-stack interval). Image analysis was performed using ImageJ software. Immunostained structures were counted as NMJs when SV2 was opposed to α-bungarotoxin with at least 50% co-localization. The outer perimeter of each area of co-localization on the horizontal myoseptum was drawn by hand and the enclosed area was measured. The area of each myotome was also measured, and the area of co-localization was measured as a fraction of the myotome it occupied. For AChR quantification, AChR clusters were thresholded and their area and intensity measured, and the number AChR clusters greater than 20 μm^2 in size per myotome were compared. For prepatterned AChR quantification, the five caudal-most myotomal segments were compared in 17 hpf embryos, and the number of AChRs greater than 3 μm^2 per myotome were quantified. Axon pathfinding defects were quantified by counting the percentage of myotomes with axons which failed to cross the horizontal midline at 24 hpf. When comparing values from treated zebrafish with values from untreated zebrafish an unpaired Student's *t*-test or chi-square test was used. Thresholds of $P < 0.05$ were considered significant. We confirmed normal distributions of data before performing parametric tests.

Conflict of Interest statement. None declared.

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General Article

GENERAL ARTICLE

Salbutamol modifies the neuromuscular junction in a mouse model of ColQ myasthenic syndrome

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Abstract

The β-adrenergic agonists salbutamol and ephedrine have proven to be effective as therapies for human disorders of the neuromuscular junction, in particular many subsets of congenital myasthenic syndromes. However, the mechanisms underlying this clinical benefit are unknown and improved understanding of the effect of adrenergic signalling on the neuromuscular junction is essential to facilitate the development of more targeted therapies. Here, we investigated the effect of salbutamol treatment on the neuromuscular junction in the ColQ deficient mouse, a model of end-plate acetylcholinesterase deficiency. ColQ$^{-/-}$ mice received 7 weeks of daily salbutamol injection, and the effect on muscle strength and neuromuscular junction morphology was analysed. We show that salbutamol leads to a gradual improvement in muscle strength in ColQ$^{-/-}$ mice. In addition, the neuromuscular junctions of salbutamol treated mice showed significant improvements in several postsynaptic morphological defects, including increased synaptic area, acetylcholine receptor area and density, and extent of postjunctional folds. These changes occurred without alterations in skeletal muscle fibre size or type. These findings suggest that β-adrenergic agonists lead to functional benefit in the ColQ$^{-/-}$ mouse and to long-term structural changes at the neuromuscular junction. These effects are primarily at the postsynaptic membrane and may lead to enhanced neuromuscular transmission.

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Introduction

Motor neurons contact their target muscle fibres at highly specialised chemical synapses, neuromuscular junctions (NMJ). The NMJ is the pathogenic target in a wide range of human diseases, including those resulting from genetic defects affecting a diverse range of proteins which are critical for synaptic function, the Congenital Myasthenic Syndromes (CMS) (1,2). CMS arise from mutations affecting presynaptic, synaptic or postsynaptic proteins at the NMJ, resulting in impairment of neuromuscular transmission by one or more mechanisms. A precise molecular classification of CMS subtype is of great importance for the diagnosis and genetic counselling of patients, but also to allow administration of effective treatment as different drugs may be beneficial or deleterious depending on the CMS subtype (3). For many subtypes, clinical benefit is gained from acetylcholinesterase (AChE) inhibitors, which augment the synaptic response to acetylcholine (ACh) (4). However, AChE inhibitors are ineffective or even detrimental in Dok7 CMS, slow-channel CMS, end-plate AChE deficiency and MuSK CMS.

Ephedrine, a sympathomimetic with α- and β-adrenergic effects, and salbutamol, a selective β_2–agonist, have been successfully used to treat many patients with CMS subtypes which are not effectively treated by anticholinesterases. These include those with mutations that cause deficits in Dok-7, Agrin, MuSK, ALG2, AChR (ε -subunit) and in end-plate AChE deficiency (5–10). In contrast with the effects of anticholinesterases, the full effects of these adrenergic treatments are not immediate, reaching a peak only after several months (5,11). Among the varied pharmacologic effects of β-agonists, there is considerable evidence for their numerous effects in regulating skeletal muscle structure and function, and in exerting an anabolic effect on skeletal muscle protein metabolism (12–16). These actions are predominantly mediated through the β_2 receptors (ADBR2), and involve cAMP signalling (17). While these effects were initially exploited by the livestock industry, their use quickly expanded to include body builders and athletes. In more recent years, experimental interest has further expanded to trial the treatment of a wide range of muscle-wasting and neuromuscular diseases. In animal and human studies, β_2-adrenergic agonists have been reported to have a positive but limited effect in dystrophic and injured muscle, as well as in congenital myopathies and fascioscapulohumeral muscular dystrophy (18–21). In addition, further studies have suggested β_2-agonists may have a modest effect in spinal muscular atrophy, and in denervated muscle following spinal cord injury (22,23).

It is in treatment of CMS however, that sympathomimetics have demonstrated conclusive clinical benefit, and they now comprise standard treatment for some subtypes of CMS in the form of oral ephedrine or salbutamol (24). Several observational studies demonstrate improvements in motor symptoms and timed tests when CMS patients are administered ephedrine or salbutamol and mobility may often improve to the extent of regaining ambulation in wheelchair bound patients (7,9–11,25–27). However, it is not known why treatments acting via pathways mediated by the sympathetic nervous system have therapeutic benefit in disorders of the NMJ. Despite its potential implications in the understanding of both the pathogenesis and treatment of many neuromuscular diseases, the effect of β-agonists on the maturation and maintenance of NMJs has never been clearly defined.

In order to address this, we have studied the effect of salbutamol treatment on a model of end plate AChE deficiency, the ColQ knockout (ColQ$^{-/-}$) mouse. In these mice, endplate AChE deficiency is caused by mutations not in *ACHE* itself but in *COLQ*, which encodes the collagenic tail subunit bound to the catalytic subunit in the asymmetric AChE found at the skeletal NMJ (28,29). The N-terminal domain of ColQ anchors asymmetric AChE to the synaptic basal lamina (30). In humans, mutations in *COLQ* typically result in fatigable muscle weakness presenting in the neonatal period or early infancy, often accompanied by episodes of respiratory failure (31). The NMJs of these patients have abnormalities in both the function (prolonged response to ACh due to the absence of AChE activity) and structure (disrupted postsynaptic apparatus, probably resulting from excessive Ca^{2+}entry into the muscle through the AChRs) (32,33).

Generally, these patients show either no long-term benefit or worsening of symptoms with AChE inhibitors. However, treatment with salbutamol and ephedrine has been shown to lead to improved mobility and respiratory function in these patients (25). ColQ$^{-/-}$ mice also lack all asymmetric AChE and exhibit both muscle weakness and abnormalities of the NMJ (34,35), thus closely resembling the human disease. In addition to its role in anchoring AChE, ColQ binds to the key postsynaptic organiser protein MuSK, and ColQ has also been shown to have an important regulatory role in postsynaptic development and maturation through this interaction (36–38). Given the clinical improvement from salbutamol treatment in CMS patients is gradual, requiring a period of weeks to months for full benefit, we have tested the effects of long-term administration of salbutamol, i.e. administered over a period of 7 weeks, in the ColQ$^{-/-}$ mouse.

Here, we show that salbutamol treatment leads to a gradual improvement in muscle strength in the ColQ$^{-/-}$ mouse. In addition, we show that morphological defects of the NMJ in the ColQ$^{-/-}$ mouse can be partially rescued by salbutamol treatment, in ways which are likely to lead to enhanced neuromuscular transmission. These results provide new evidence for the long-term effects of adrenergic signalling on the structural properties of the NMJ.

Results

Salbutamol treatment improves muscle strength in ColQ$^{-/-}$ mice

From 3 weeks of age, ColQ$^{-/-}$ mice received daily subcutaneous injections of salbutamol or vehicle control (water) for 7 weeks. Previous work has shown that ColQ$^{-/-}$ mice exhibit muscle weakness which is apparent from P5 (34). We used forelimb grip strength to assess muscle strength at 3 time points (Fig. 1A) in wild type (WT), ColQ$^{-/-}$ mice treated with water and ColQ$^{-/-}$ mice treated with salbutamol. The performance of water treated ColQ$^{-/-}$ mice was significantly worse than WT littermates at baseline (3 weeks old) and at subsequent measurements (6 weeks old and 9 weeks old). In comparison, ColQ$^{-/-}$ mice treated with salbutamol showed a gradual improvement in grip strength, which became significantly better than water treated ColQ$^{-/-}$ mice at 9 weeks (after 6 weeks of salbutamol treatment) (water treated ColQ$^{-/-}$ 41.6 ± 11.7 g vs. salbutamol treated ColQ$^{-/-}$ 61.9 ± 7.4 g (mean ± S.D.)). ColQ$^{-/-}$ mice were also smaller than WT littermates, having a body weight approximately 50% that of littermates at 3 weeks (Fig. 1B) (34). This low body weight persisted into adulthood, and was not altered by salbutamol, with no significant difference in salbutamol treated ColQ$^{-/-}$ mice body weight during 7 weeks of salbutamol treatment (water treated ColQ$^{-/-}$ 21.3 ± 2.71 g vs. salbutamol treated ColQ$^{-/-}$ 19.5 ± 2.13 g). In summary, salbutamol led to a gradual

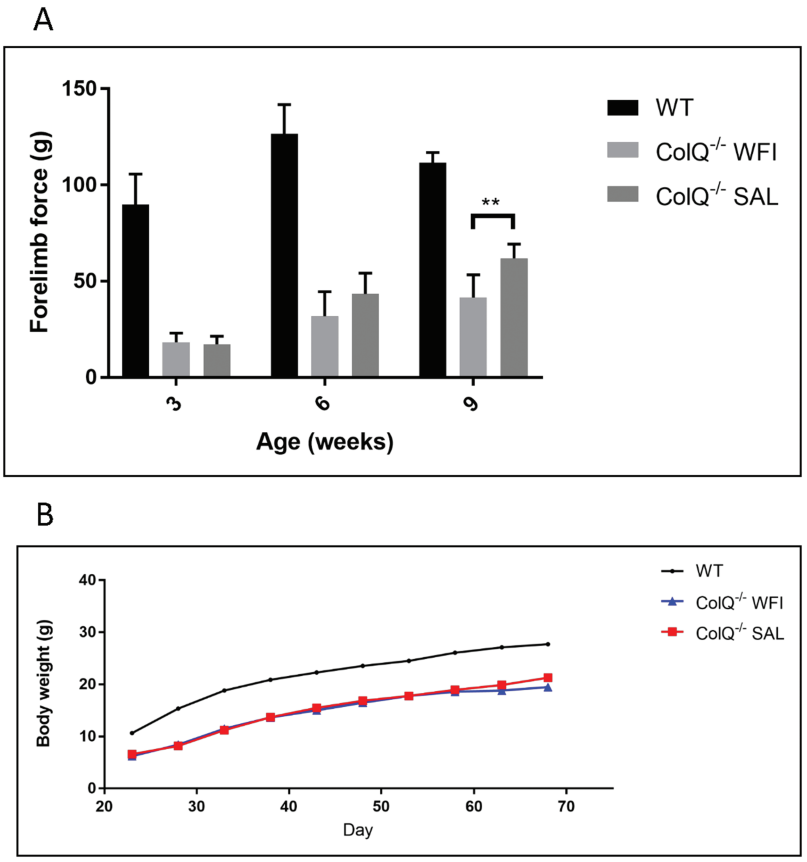

Figure 1. Salbutamol treatment improves muscle strength in ColQ$^{-/-}$ mice but does not alter body weight. **A.** Forelimb grip strength of WT, water treated ColQ$^{-/-}$ (ColQ$^{-/-}$ WFI) and salbutamol treated ColQ$^{-/-}$ (ColQ$^{-/-}$ SAL) mice measured at 3, 6 and 9 weeks of age. Grip strength is significantly impaired in water treated ColQ$^{-/-}$ mice compared to WT littermates at baseline, and at subsequent time points. Salbutamol treated ColQ$^{-/-}$ mice showed a tendency towards improved grip strength after 3 weeks of treatment (6-wks-old) which became statistically significant after 6 weeks of treatment (9-wks-old). n = 6 animals per group. Error bars depict S.D. ** indicates $P < 0.01$. **B.** Growth curve demonstrating changes in body weight over 7 weeks of salbutamol treatment. ColQ$^{-/-}$ mice are significantly smaller than WT littermates at all time-points and body weight is not affected by salbutamol treatment. n = 6 animals per group.

improvement in limb muscle strength but did not affect body weight in ColQ$^{-/-}$ mice.

Salbutamol treatment improves NMJ structural defects in ColQ$^{-/-}$ mice

It has previously been shown that the ColQ$^{-/-}$ mouse exhibits abnormal synaptic structure, including NMJs which appear fragmented or immature (34). We studied the pre and postsynaptic morphology of NMJs from 10 week old ColQ$^{-/-}$ lumbrical muscles and compared these to salbutamol treated ColQ$^{-/-}$ muscles, using a standardised morphometric analysis platform ('NMJ-morph') (39). ColQ$^{-/-}$ mice did demonstrate presynaptic structural defects, with small but significant decreases in axon diameter (WT 3.49 ± 0.79 µm vs water treated ColQ$^{-/-}$ 2.90 ± 1.47 µm) and nerve terminal area (WT 227.16 ± 35.12 µm^2 vs water treated ColQ$^{-/-}$ 166.28 ± 47.16 µm^2) (Fig. 2B). These presynaptic defects were not significantly different in salbutamol treated ColQ$^{-/-}$ mice (axon diameter and nerve terminal area 2.96 ± 1.43 µm and 159.42 ± 33.89 µm^2 respectively).

Postsynaptic morphology was more obviously perturbed in the ColQ$^{-/-}$ mice (Fig. 2C-E and Supplementary Material, Fig. S1), with ColQ$^{-/-}$ lumbrical muscles showing significantly reduced AChR area (WT 332.117 ± 58.55 µm^2 vs. water treated ColQ$^{-/-}$ 236.91 ± 63.13 µm^2) and reduced NMJ 'compactness' (derived from (AChR Area/Endplate area) x100), a measure of AChR dispersal (WT 71.77 ± 13.49% vs. water treated ColQ$^{-/-}$ 54.30 ± 19.01%) (39,40). These postsynaptic defects were significantly improved in salbutamol treated mice (AChR area 303.52 ± 75.69 µm^2 and compactness 76.23 ± 22.26%).

Furthermore, the area of synaptic contact (the area of contact between pre and postsynaptic NMJ components) was significantly reduced in the ColQ$^{-/-}$ mice compared to WT littermates (Fig. 2D) (WT 191.61 ± 46.69 µm^2 vs. water treated ColQ$^{-/-}$ 93.8 ± 64.98 µm^2), and this was increased by salbutamol treatment (141.63 ± 58.65 µm^2). In keeping with previous studies, we found the number of discrete fragments of AChR rich membrane was significantly increased in the ColQ$^{-/-}$ mice (WT 2.88 ± 0.93 fragments vs. water treated ColQ$^{-/-}$ 4.10 ± 1.74 fragments). Fragmentation was not altered by salbutamol treatment, however (3.91 ± 1.00 fragments). We also examined these parameters in soleus muscles (Supplementary Material, Fig. S1). Salbutamol treatment also improved AChR area, compactness and area of synaptic contact in soleus muscles, and we found no difference in the magnitude of the effect of salbutamol in soleus muscle compared to lumbricals (Supplementary Material, Fig. S1).

These results prompted us to examine whether the fluorescence intensity per µm^2 of α-BTX, a measure of the local AChR density, was different in salbutamol treated mice. In lumbricals from water treated ColQ$^{-/-}$ mice, AChR density was significantly reduced compared to WT littermates (mean pixel intensity per µm^2 WT 255.39 ± 149.49 vs. water treated ColQ$^{-/-}$ 119.68 ± 69.49).

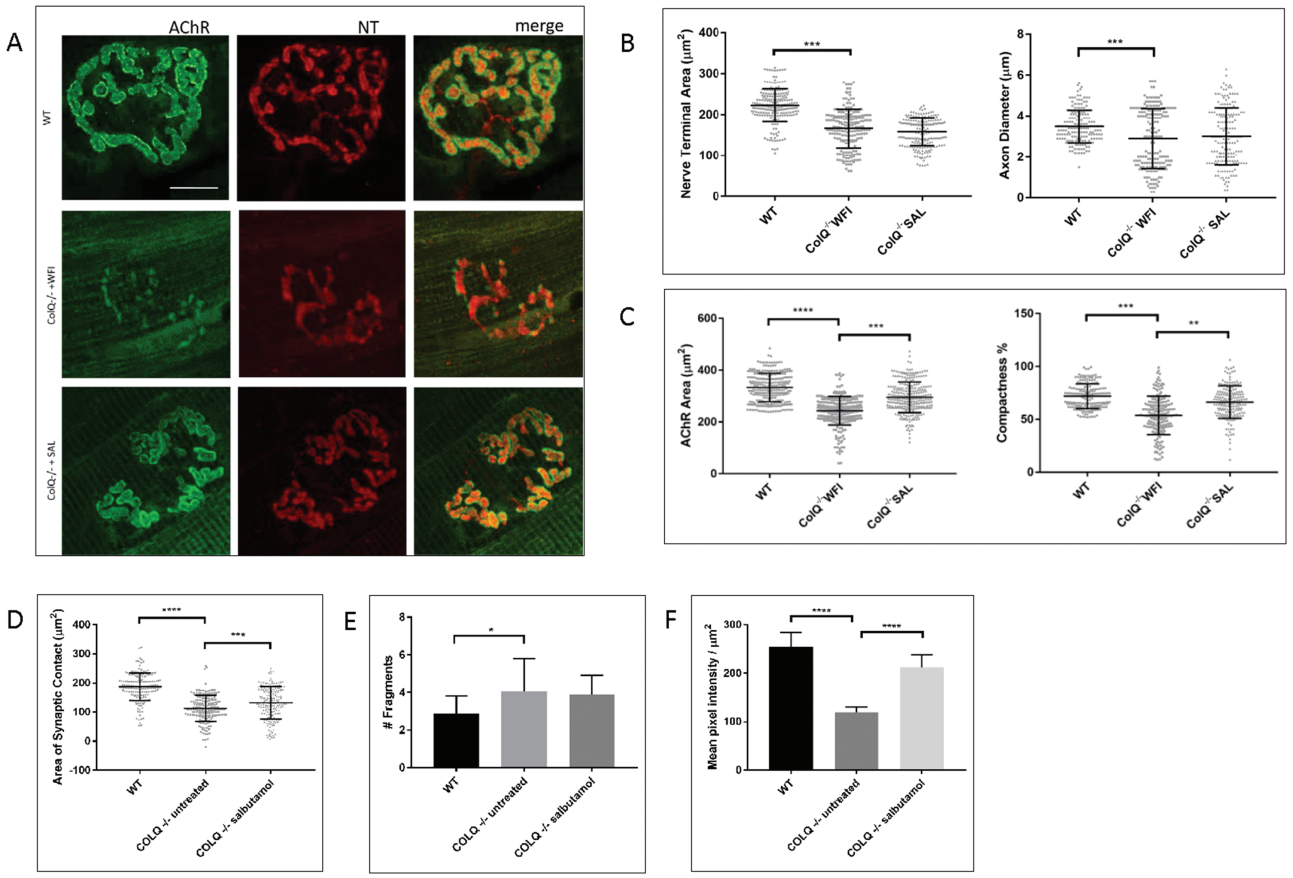

Figure 2. Salbutamol treatment improves postsynaptic NMJ morphology in ColQ$^{-/-}$ mice. **A.** Representative confocal micrographs of NMJs in the lumbrical muscles of 10-wk-old WT, water treated ColQ$^{-/-}$ (ColQ$^{-/-}$ WFI) and salbutamol treated ColQ$^{-/-}$ (ColQ$^{-/-}$ SAL) mice, labelled with anti-neurofilament (red) and anti-synaptophysin (red, both NT) and Alexa fluor 488 α-bungarotoxin (green, AChR). Water treated ColQ$^{-/-}$ mice display variable NMJ morphology with smaller NMJs with reduced AChR density. Scale bar, 10 µm. **B.** Quantitative analysis of presynaptic variables from lumbrical muscles. ColQ$^{-/-}$ mice exhibited smaller nerve terminals with thinner axons, and these variables were unchanged in salbutamol treated mutants. **C.** Quantitative analysis of postsynaptic variables from lumbrical muscles. Water treated ColQ$^{-/-}$ mice had reduced AChR area and compactness, which were significantly increased in salbutamol treated ColQ$^{-/-}$ mice compared to water treated ColQ$^{-/-}$ mice. **D.** Quantitative analysis of area of synaptic contact. ColQ$^{-/-}$ mice have significantly reduced area of synaptic contact which is increased by salbutamol treatment but remains smaller than in WT animals. **E.** Quantitative analysis of fragmentation of NMJs. ColQ$^{-/-}$ NMJs exhibit significantly increased number of AChR rich fragments per NMJ, and this was not different in salbutamol treated animals. n = 180 NMJs analysed per group for B-E. Analysis of NMJ-morph variables from soleus muscle are provided in Supplementary Material, Figure S2. **F.** Quantification of α-bungarotoxin fluorescence intensity of individual NMJs measured from projection of confocal stacks from lumbrical muscles. Fluorescence intensity was significantly decreased in water treated ColQ$^{-/-}$ mice, and was significantly increased in salbutamol treated ColQ$^{-/-}$ mice compared to water treated ColQ$^{-/-}$ mice. n = 120 NMJs analysed per group for F. All error bars depict S.D. Unpaired Student's *t* tests. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

In comparison, in salbutamol treated ColQ$^{-/-}$ lumbricals, AChR density was significantly increased compared to water treated ColQ$^{-/-}$ mice (mean pixel intensity per µm^2 211.89 ± 139.41).

Together, these observations suggest that salbutamol treatment can alter NMJ structural defects in ColQ$^{-/-}$ mice, and in particular increase AChR area, synaptic area and AChR density.

Salbutamol alters ultrastructural appearances of the postsynaptic membrane

In mature NMJs, the postsynaptic membrane is invaginated with extensive folds extending into the postsynaptic membrane, which amplify the transmitter action of ACh (41). AChRs accumulate at the crests of these postjunctional folds, which can be visualised at the EM level by electron dense material at the top of the folds and extending partly down the folds (42). Under the electron microscope, many NMJs from intercostal muscles of 10 week old water treated ColQ$^{-/-}$ mice appeared normal, as has been previously reported in 6 month old ColQ$^{-/-}$ mice (34). However, in 50% of NMJs the extent of folding appeared clearly reduced and regions of high electron-density at the crests of folds were lost (Fig. 3A). We quantified the extent of postjunctional folding using fold index (total surface length of postsynaptic membrane measured along the tops of the folds/total length of postsynaptic membrane including folds) a measure of the increase in postsynaptic membrane area resulting from folding (43). This revealed a significant reduction in extent of folding in ColQ$^{-/-}$ mice compared to WT (mean fold index WT 4.28 ± 1.38 vs. water treated ColQ$^{-/-}$ 2.50 ± 0.72) (Fig. 3B). In addition, in salbutamol treated ColQ$^{-/-}$ mice, fold index was significantly increased compared to water treated ColQ$^{-/-}$ mice (3.65 ± 1.03). Additional features measured from EM images are provided in Supplementary Material, Table S1.

Immunoreactivity of Agrin, Dystroglycan and MuSK in ColQ$^{-/-}$ mice

The effects of salbutamol on postsynaptic structural defects prompted us to explore the effect of salbutamol on MuSK, agrin

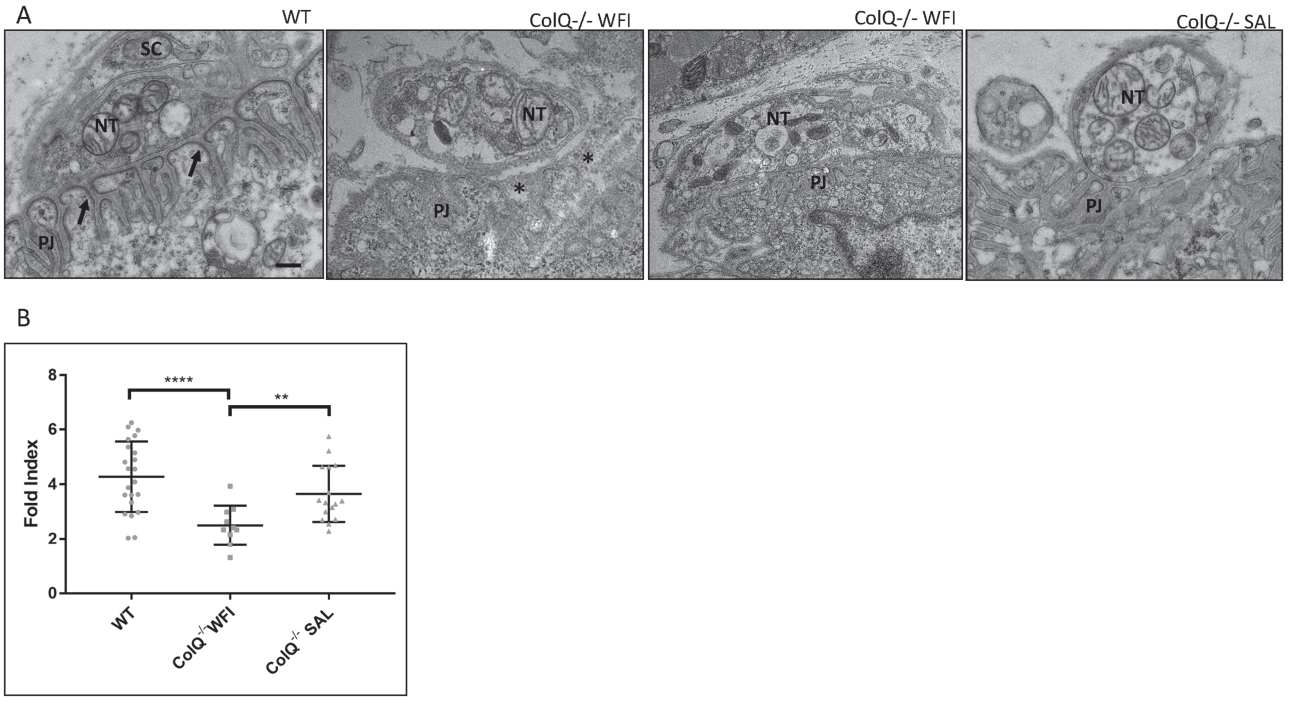

Figure 3. The extent of postjunctional folding is increased in salbutamol treated ColQ$^{-/-}$ mice. A. Representative electron micrographs of sections through single boutons from intercostal muscles of 10-wk-old WT, water treated ColQ$^{-/-}$ (ColQ$^{-/-}$ WFI) and salbutamol treated ColQ$^{-/-}$ (ColQ$^{-/-}$ SAL) mice. NT: nerve terminal, PJ: postjunctional folds, SC; Schwann cell. In WT mice, the postsynaptic membrane is extensively infolded and AChRs can be seen as areas of high electron-density at the crests of the folds (arrows). In ColQ$^{-/-}$ mice, the appearance of increased membrane density at the tops of the folds is lost, the extent of folding is reduced and the synaptic space appears widened (stars, right panel) although some NMJs appear normal (left panel). Salbutamol treated ColQ$^{-/-}$ NMJs exhibit similar ultrastructural appearances to WT littermates. Scale bar 0.5 µm. B. Quantitative analysis of changes in the extent of folding with salbutamol treatment. In salbutamol treated ColQ$^{-/-}$ mice, the extent of postjunctional folding is significantly increased compared to water treated ColQ$^{-/-}$ mice, as measured by fold index (fold length/postsynaptic length). n ≥ 15 boutons from three mice analysed per group. Mann Whitney *U* test. ****$p < 0.0001$, **$p < 0.01$.

and α-dystroglycan. MuSK provides the primary scaffold for AChR clustering and postsynaptic differentiation (44). The C-terminus of ColQ binds MuSK and ColQ deficiency leads to reduced levels of membrane bound MuSK (36,37). The glycoprotein agrin is secreted by motor axon terminals to activate the LRP4/MuSK/Dok7 complex to induce and stabilise AChR clusters (45). Dystroglycan, a component of the dystrophin associated glycoprotein complex (DGC), is essential for the assembly of a synaptic basement membrane, and linking the extracellular matrix to the cytoskeleton (46). ColQ binds to perlecan which in turn binds α-dystroglycan, and this interaction is necessary for the synaptic localisation of AChE (47). In addition, the localisation of AChRs at the crests of the folds arises through their interaction with the DGC (48).

In order to determine membrane bound MuSK protein levels, we analysed MuSK fluorescence intensities per AChR cluster in transverse sections of gastrocnemius muscle from WT, water treated ColQ$^{-/-}$ and salbutamol treated ColQ$^{-/-}$ mice (Fig. 4A). MuSK fluorescence intensity was significantly reduced in water treated ColQ$^{-/-}$ mice compared to WT (mean MuSK-to-α-BTX fluorescence intensity ratio WT 0.76 ± 0.21 vs. water treated ColQ$^{-/-}$ 0.44 ± 0.06). In salbutamol treated ColQ$^{-/-}$ muscle, the MuSK-to-α-BTX fluorescence intensity ratio was increased compared to water treated ColQ$^{-/-}$ mice (Fig. 4B, mean MuSK-to-α-BTX fluorescence intensity ratio 0.55 ± 0.12 in salbutamol treated ColQ$^{-/-}$ mice). In contrast to MuSK staining, the fluorescence intensity of agrin was not significantly different in either water treated ColQ$^{-/-}$ or salbutamol treated ColQ$^{-/-}$ mice compared to WT animals (data not shown). In addition, the distribution and intensity of immunostaining of α-dystroglycan was qualitatively similar in ColQ$^{-/-}$ and WT mice, and was not affected by salbutamol treatment (data not shown).

Salbutamol treatment does not alter muscle fibre area or fibre type composition in ColQ$^{-/-}$ mice

It has previously been shown that ColQ$^{-/-}$ mice exhibit both reduced muscle fibre diameter and alteration of fibre type composition (35,38). We therefore examined the effect of salbutamol treatment on these parameters. At 10 weeks, muscle fibre cross sectional area in water treated ColQ$^{-/-}$ mice was significantly reduced in both gastrocnemius (mean cross sectional area WT 1740 ± 434.73 µm^2 vs. water treated ColQ$^{-/-}$ 1342 ± 317.51 µm^2) and soleus (WT 1188 ± 105.4 µm^2 vs water treated ColQ$^{-/-}$ 781 ± 185.44 µm^2). Muscle fibre area was not significantly different in salbutamol treated ColQ$^{-/-}$ mice compared to water treated ColQ$^{-/-}$ mice (Fig. 5A and B) (mean cross sectional area 1308 ± 350.45 µm^2 and 802 ± 217.51 µm^2 in gastrocnemius and soleus muscles respectively). In addition, examination of fibre type composition in gastrocnemius and soleus muscles revealed a dramatic reduction in MHC type 1 expressing fibres in both muscles, as well as an increase in MHC type 2A fibres in water treated ColQ$^{-/-}$ mice. Again, these changes in fibre type composition were unaffected by salbutamol treatment (Fig. 5C-F). These data indicate that the effect of salbutamol on NMJ structure and on grip strength in the ColQ$^{-/-}$ mouse is not secondary to changes in skeletal muscle trophism.

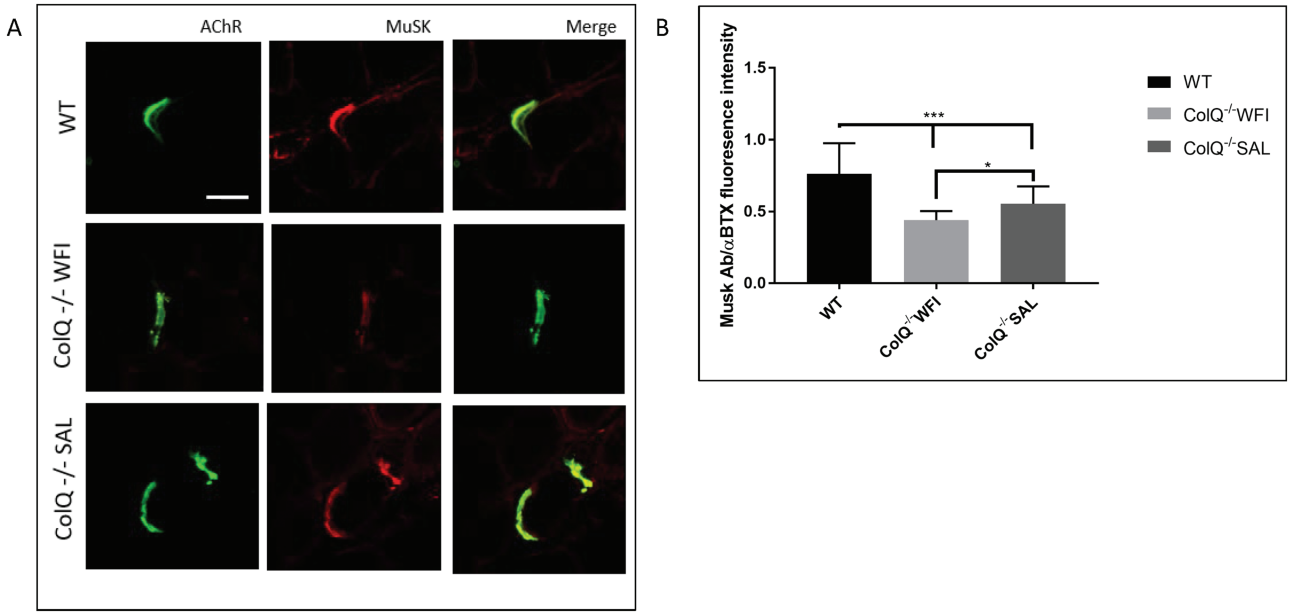

Figure 4. Salbutamol treatment alters MuSK immunoreactivity in ColQ$^{-/-}$ mice. **A.** AChR and MuSK clusters labelled with Alexa 488 α-BTX (green, AChR) and anti-MuSK antibody (red) respectively on transverse sections of gastrocnemius muscle from 10-wk-old WT, water treated ColQ$^{-/-}$ (ColQ$^{-/-}$ WFI) and salbutamol treated ColQ$^{-/-}$ (ColQ$^{-/-}$ SAL) mice. Scale bar, 20 μm. **B.** Quantification of MuSK fluorescence intensities from projection of confocal stacks. MuSK fluorescence intensity per AChR cluster was decreased in ColQ$^{-/-}$ muscle, and significantly increased in salbutamol treated ColQ$^{-/-}$ mice compared to water treated. n = 180 NMJs per group for B. Unpaired Student's *t* tests. ***$p < 0.001$, *$p < 0.05$, n.s. not significant.

Immunoreactivity of β2 adrenoceptors colocalises with the NMJ in WT and ColQ$^{-/-}$ mice

Salbutamol is a selective ADBR2 agonist, and it has been previously shown that ADBR2 is the predominant adrenoceptor subtype in skeletal muscle (49,50). In order to investigate the distribution of ADBR2 on the muscle membrane we stained transverse sections of gastrocnemius muscle with ADBR2 antibody and α-BTX (Fig. 6A). This revealed co-localisation of ADBR2 immunoreactivity at the NMJ. In addition, this pattern of immunostaining did not appear to be altered in ColQ$^{-/-}$ mice and was not affected by 7 weeks of salbutamol treatment (Fig. 6B).

Discussion

Our study provides evidence that treatment of AChE-deficient ColQ$^{-/-}$ mice with the sympathomimetic salbutamol, over a period of weeks, partially normalises both their weakness of grip strength and the structural abnormalities of NMJs that may help to explain that weakness. It has long been suspected that activation of adrenergic receptors can alter neuromuscular transmission. *In vitro*, ephedrine increases quantal content, and ephedrine and salbutamol block the AChR channel, although only when applied at higher concentrations than obtained at therapeutic doses in humans (51,52). In addition, adrenaline and noradrenaline can potentiate neuromuscular transmission, and activation of α_1 and β adrenoceptors has been shown to enhance nerve evoked ACh release (53–55). However, these immediate effects do not account for the delayed therapeutic action of salbutamol in patients with CMS, which suggest an additional longer term modulation of NMJ function. There is, however, increasing evidence for the role of the sympathetic nervous system in maintenance of the NMJ. Recent studies demonstrate that NMJs are intimately linked to a network of sympathetic neurons within skeletal muscle which increase during postnatal development, a network which is critical for the morphological integrity of the NMJ (56,57). We previously showed that salbutamol rescued aberrant NMJ development in zebrafish embryos lacking Dok7 and MuSK (58). Here, in a setting more closely resembling human disease and treatment regimens, we show that long-term administration of salbutamol leads to functional benefit and improves postsynaptic structural defects in a mouse model of end plate AChE deficiency.

Salbutamol treatment resulted in a significant increase in grip strength of ColQ$^{-/-}$ mice. This effect was gradual, becoming significantly better only after 6 weeks of daily administration. This mirrors the effect in humans with ColQ CMS where, unlike the rapid clinical benefit seen from AChE inhibitors in other forms of CMS, the response to sympathomimetics is more gradual with an increasingly positive response over 3–6 months of treatment (11,59). As previously reported, the phenotype of the ColQ$^{-/-}$ mouse is not as severe as would be expected from a complete lack of AChE. Interestingly, in both the salbutamol treated and water treated ColQ$^{-/-}$ mice, grip strength was improved at 9 weeks compared to 3 weeks. In previous studies of the ColQ$^{-/-}$ mice, NMJ abnormalities also appeared to lessen with age, suggesting a compensatory mechanism (34). These studies suggested that capping of the motor nerve terminal by the Schwann cell serves to protect the end-plate from lost ACh clearance (34,60). However, invasion of the synaptic cleft by the Schwann cell was not a feature in any of the NMJs examined with EM in this study. The findings may reflect the homeostatic plasticity of the NMJ, as occurs in response to trauma, toxins and in autoimmune myasthenia gravis (41,61,62). The precise processes underlying adaptive plasticity in the ColQ$^{-/-}$ mouse have not yet been determined.

The effects of β-adrenergic agonists on skeletal muscles are known to include muscle fibre hypertrophy and alteration of fibre type composition (63–65). The ColQ$^{-/-}$ mouse exhibits features of muscle atrophy, with reduction in type I and increase

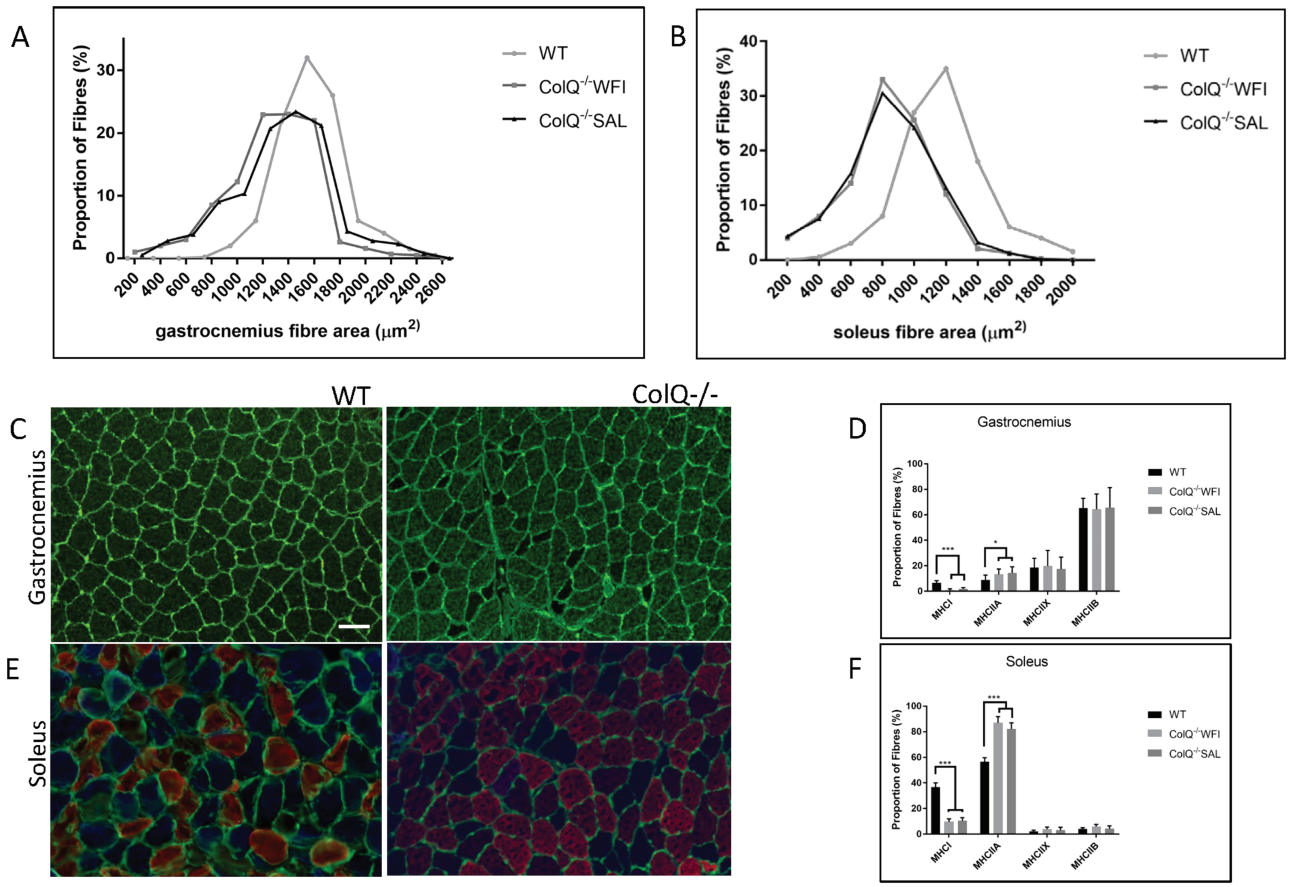

Figure 5. Alterations in muscle fibre diameter and type in ColQ$^{-/-}$ mice are not affected by salbutamol treatment. **A, B:** Frequency distributions of muscle fibre cross sectional area from 10-wk-old WT, water treated ColQ$^{-/-}$ (ColQ$^{-/-}$ WFI) and salbutamol treated ColQ$^{-/-}$ (ColQ$^{-/-}$ SAL) gastrocnemius (A) and soleus (B) muscles. Plots represent percentage of fibres in each size bin. Fibres are significantly smaller in ColQ$^{-/-}$ muscles compared with those in WT fibres (Mann-Whitney *U* test) but fibre diameter was unaffected by 7 weeks of salbutamol treatment. n = 6 muscles per genotype, with ≥500 muscle fibres sampled to generate the size distributions. ***$p < 0.001$, *$p < 0.05$ **C—F:** Fibre type composition in WT and ColQ$^{-/-}$ muscles. Transverse sections of gastrocnemius (C) and soleus (E) muscles from 10-wk-old WT and ColQ$^{-/-}$ animals, stained with anti-laminin (green), to delineate the fibre circumference and MHC Type 1 (blue), Type IIa (red) and Type IIb (green). ColQ$^{-/-}$ mice exhibit significantly fewer Type 1 fibres and increased Type 2A fibres in both gastrocnemius (D) and soleus (F) muscles, which was not affected by 7 weeks of salbutamol treatment. Scale bar, 50 μm.

in type IIA fibres as well as reduced fibre size (38). It is plausible therefore, that adrenergic agonists lead to clinical benefit in ColQ CMS due to promotion of muscle growth. However, our analysis of fibre type composition and area in both soleus and gastrocnemius muscles revealed no detectable effect of salbutamol treatment on any parameter measured in ColQ$^{-/-}$ mice. This suggests that the improved grip strength in salbutamol treated ColQ$^{-/-}$ animals cannot be readily explained by an anabolic effect on skeletal muscle. This is consistent with the lack of evidence of clinical benefit of sympathomimetic treatment in myopathies and muscular dystrophies (20,21,66,67). Since both the structure and function of the NMJs is abnormal in ColQ$^{-/-}$ mice (34), we turned our attention to the possibility that salbutamol may act to enhance muscle activation by action on the NMJs.

In support of this view, we found evidence of a partial normalisation of synaptic area, local AChR density and the extent of postsynaptic folding in salbutamol treated ColQ$^{-/-}$ mice, all changes which would be likely to enhance the efficacy of neuromuscular transmission. Using standardised analysis of NMJs (39), we confirmed pronounced defects in the structure of the NMJs in water treated ColQ$^{-/-}$ mice which are likely to be associated with a decreased efficacy of neuromuscular transmission (34,35). These involved changes to both presynaptic and postsynaptic components of the NMJ. On the presynaptic side, there was a modest reduction in both the terminal axon diameter and nerve terminal area. There was also a significant reduction in the area of overlap between the nerve terminal and the underlying region of high AChR density ('synaptic area'). There is an approximately linear relationship between synaptic area and quantal content, and a pathological reduction in synaptic area has been demonstrated in patients with ColQ CMS, and in CMS due to mutations in the postsynaptic adaptor *DOK7* (60,68,69). Therefore, any reduction in synaptic area is likely to be associated with reduced quantal content and the efficacy of neuromuscular transmission.

On the postsynaptic side, we found that at 10 weeks of age, the labelling intensity of AChRs was strikingly reduced in water treated ColQ$^{-/-}$ mice, suggesting a reduced local AChR density at the NMJs. Any reduction in local AChR density would be expected to reduce the number of AChRs opened by individual transmitter quanta and thus impair neuromuscular transmission. However, previous studies of ColQ$^{-/-}$ mouse showed an increased AChR density in sternomastoid and soleus muscles at 7 days of age (36). These divergent findings may be due to differences in the age of the mice examined, given that at postnatal day 7 mouse NMJs are still undergoing structural and molecular maturation and AChR

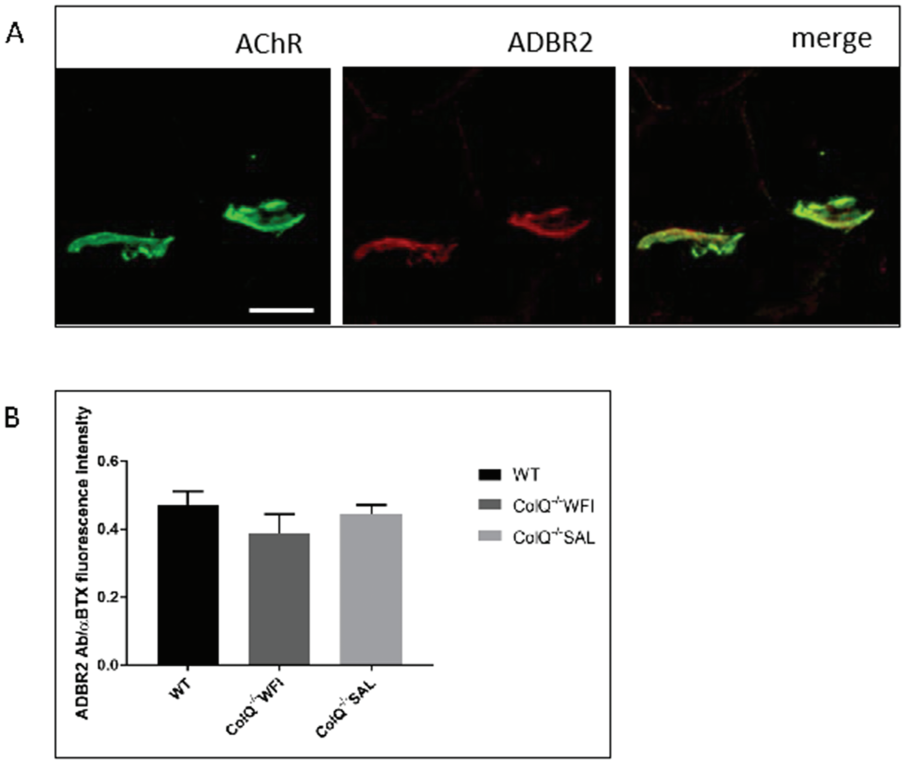

Figure 6. β_2 adrenergic receptor immunostaining co-localises with the NMJ. **A.** Transverse sections of gastrocnemius muscle from 10-wk-old WT mice stained with anti-ADBR2 antibody (red) and Alexa 488 α-BTX (green, AChR). ADBR2 staining is enriched at the NMJ. Scale bar, 20 μm. **B.** Quantification of ADBR2 fluorescence intensities per AChR cluster from projection of confocal stacks. ADBR2 fluorescence intensity per AChR cluster was not significantly different between WT, water treated ColQ$^{-/-}$ or salbutamol treated ColQ$^{-/-}$ mice. Unpaired Student's *t* tests.

rich membrane is not yet fully restricted to regions of motor axon contact (70). AChR deficiency has also been shown in ColQ CMS patients (60). In parallel with the reduction of AChR density, we found a decrease in the area occupied by a high density of AChRs. This extends previous studies showing reduced AChR area in adult (diaphragm, levator auris longis) and 7 days old (soleus and sternomastoid) ColQ$^{-/-}$ mice (35,36).

A likely explanation for reduced AChR density at the NMJ is the simplification and disorder of the postsynaptic folds associated with AChE deficiency (32). Since a substantial fraction of AChRs are normally present in the membrane of the folds closest to the nerve, any disruption of membrane folding may result in reduced numbers of AChRs as detected by fluorescence microscopy. Consistent with this view, our preliminary analysis of NMJs from intercostal muscles of ColQ$^{-/-}$ mice confirmed the significant reduction in the extent and orderliness of postjunctional folds. The high density of voltage-gated sodium channels in the depths of the folds is believed to facilitate initiation of the muscle fibre action potential (69,71), suggesting a second way in which the reduced folding in ColQ$^{-/-}$ mice may impair neuromuscular transmission.

Treatment of ColQ$^{-/-}$ mice with salbutamol for 7 weeks resulted in a partial normalisation of a number of the abnormalities of NMJ structure which are likely to be associated with impaired NMJ function, particularly those associated with the postsynaptic component. These include the area of synaptic contact, the local AChR density and the extent of folding, with similar degrees of effect found in lumbrical and soleus muscles. Whilst electrophysiological studies will be required in order to confirm that these structural alterations are coupled with improved NMJ function, the changes are likely to have important implications for the efficiency of neuromuscular transmission (41,72–74). Our findings of pronounced postsynaptic structural effects from salbutamol are in keeping with the fact that it is the CMS subtypes with predominant postsynaptic alterations which show greatest clinical benefit from salbutamol and ephedrine (4,7,11,75).

The differentiation of the postsynaptic region at the NMJ is strongly influenced by the activity of MuSK (44). ColQ deficiency has been shown to regulate membrane bound MuSK, and to subsequently lead to decreased signalling of MuSK as measured by reduced phosphorylation of the β-AChR subunit (36). This led us to ask whether salbutamol treatment might enhance the expression of membrane bound MuSK in ColQ−/− mice. We observed reduced MuSK immunoreactivity in gastrocnemius muscle from untreated ColQ$^{-/-}$ mice, and this was significantly increased by salbutamol treatment. It is not possible to say whether salbutamol alters the activity of the MuSK signalling pathway, or whether this finding is secondary to improved postsynaptic architecture in salbutamol treated muscles. Further studies will be required to determine whether salbutamol affects MuSK phosphorylation and kinase activity.

The distribution of ADBR2 on skeletal muscle membrane has not been previously well described. Here we observed ADBR2 immunostaining precisely co-localising with AChRs in gastrocnemius muscle from both WT and mutant adult mice, similar to the patterns previously observed in one study of mouse extensor digitorum longus muscle (56). This co-localisation is intriguing and further suggests that ADBR2 and their signalling components have an important contribution to NMJ function.

In some cases of CMS, the clinical benefit from salbutamol seems to attenuate after years of treatment (unpublished

observations). This may be due to desensitisation of ADBR2 after chronic agonist administration, as occurs when β_2-adrenergic agonists are used in the treatment of chronic heart failure and asthma, although this has not been confirmed in CMS (76,77). The development of more targeted therapies for CMS, potentially those which act downstream of ADBR2, are therefore essential to improve quality of care. We previously showed that the effects of salbutamol at NMJs of zebrafish embryos could be blocked by a selective ADBR2 antagonist, and could be replicated by directly increasing cyclic AMP with forskolin (58). Identification of the cellular processes which are regulated by the cAMP and the cAMP-dependent protein kinase (PKA) signalling pathway at the NMJ is required for the identification of possible therapeutic targets.

Postsynaptic differentiation is controlled by factors released from motor nerves, glial cells and by intrinsic muscle signalling. There are thus a multitude of pathways through which activation of ADBR2 receptors and the cAMP signalling pathway could lead to improved postsynaptic NMJ morphology (78,79). Nonetheless, our observations in the ColQ$^{-/-}$ mouse provide further evidence for important interplay between the NMJ and adrenergic signalling pathways. An understanding of the effect of sympathomimetics at the NMJ will be instrumental in order to facilitate the development of more targeted therapies, which benefit NMJ function whilst minimising systemic side-effects.

Materials and Methods

Mice

All procedures were approved by the Home Office and were carried out in accordance to the Animals Scientific Procedures Act of 1986 under project licence 70/8538. The generation of the ColQ$^{-/-}$ mouse was described by Feng et al (34). ColQ$^{+/-}$ mice were donated by the Krejci laboratory, COGNAC G cognition action group, Université Paris Descartes. Mice were bred and housed in the animal facility at the Functional Genomics Unit, Institute of Genetic Medicine, Newcastle University.

Drug treatment of mice

From post-natal day 21, ColQ$^{-/-}$ mice received daily subcutaneous injections for 7 weeks of either Salbutamol (α-[(tert-Butylamino)methyl]-4-hydroxy-m-xylene-α,α′-diol, Albuterol; Sigma) diluted in water for injection (n = 6 animals) or the equivalent volume of water alone (n = 6 animals). Injections were delivered into the loose skin over the interscapular area. Salbutamol was administered at a dose of 5 mg/kg which is equivalent to doses used in the treatment of CMS patients (59). Mice were weighed prior to injection daily. Wild type (WT) littermates were weighed and inspected daily in a similar manner to injected littermates. Researchers handling the animals were blinded to the genotype and drug allocation of each animal. Animals were weighed and inspected daily for signs of drug toxicity or side effects, and no adverse effects were observed. All mice were sacrificed within six hours of the last injection at 10 weeks of age.

Forelimb grip strength test

An electronic grip strength meter (Bioseb) was used to determine the maximal peak force of the forelimbs (80). Mice were allowed to grasp the grid and were pulled horizontally by the tail until the grip was released. The pull force was measured when the pulling force overcame the mouse's forelimb grip strength. Testing was performed in six animals per group at three time points—3 weeks, 6 weeks and 9 weeks of age. Three measurements were performed per mouse during each test and the average of these three measurements was used for statistical evaluation. These experiments were conducted in a blinded fashion by the same experimenter, 5 hours after injection.

Whole-mount muscle staining

Whole soleus and hindlimb lumbrical muscles from 10 week old mice were dissected, washed in phosphate buffered saline (PBS) for 30 minutes, and then fixed (1% paraformaldehyde in 0.1 M PBS) for 10 minutes before being teased into small bundles. After teasing, muscles were fixed in 1% PFA overnight at 4 °C. Muscles were permeabilised in ethanol followed by methanol (10 min at −20 °C each), followed by an incubation/permeabilisation step in 5% horse serum, 5% BSA, 2% Triton X-100 (for 4 hours at room temperature). Muscles were incubated overnight with antibodies against neurofilament (mouse monoclonal anti-neurofilament, 1:200, Cell Signalling technology), and synaptophysin (rabbit polyclonal anti-synaptophysin, 1:100, Thermo Fisher Scientific) or against agrin (mouse polyclonal anti-agrin, 1:250, Abcam) in PBS containing 5% horse serum, 5% BSA. The next day muscles were washed in PBS containing 5% horse serum, 5% BSA for four hours and then incubated in Alexa Fluor® 488 α-bungarotoxin (α-BTX) (1:250, Thermo Fisher Scientific), Alexa Fluor® 568 goat anti-mouse (1:250, Thermo Fisher Scientific) and Alexa Fluor® 594 goat anti-rabbit (1:250, Life Technologies) overnight. The following day muscles were washed in PBS for 4 hours and then mounted on slides with Vectashield mounting medium for fluorescence microscopy (Vector Laboratories).

NMJ imaging and analysis

Samples were visualised using a Nikon A1R laser scanning confocal microscope. Laser power and parameter settings were kept constant and Z-stack images (1-µm intervals) were acquired with x63 oil immersion objective and processed using NIS-elements AR 4.20.02 software. Soleus and lumbrical muscles from 6 mice per group were imaged; ≥30 NMJs were analysed per muscle. Variables for NMJ structural analysis were measured from maximum intensity Z-stack images using the 'NMJ-morph' protocol on ImageJ as described previously (39). For quantitation of AChR density, confocal micrographs of control and experimental mice were collected in the same session permitting comparison of fluorescence intensity. AChR density was performed only on lumbrical muscles on which NMJs could be visualised relatively quickly. The perimeters of clusters were delimited and the area and average pixel intensity calculated using ImageJ software. The AChR cluster outline was then placed in an adjacent area without clusters to record background fluorescence intensity. This reading was subtracted from the cluster reading, to give a background-corrected intensity. Lumbrical muscles from six mice per group were imaged; ≥20 NMJs were analysed per muscle.

Immunostaining on sections

Immunostaining of ADBR2, MuSK and α-dystroglycan was performed on 10 µm transverse sections of gastrocnemius

muscle cut using a cryostat (Microm HM 560, Zeiss) in the region of the motor end-plate. Sections were fixed in acetone at 4 °C for 15 minutes and then permeabilised in 0.1% Triton X-100 for 15 minutes at room temperature. Sections were blocked in PBS containing 10% goat serum, 1% BSA for 30 minutes and then incubated with primary antibody (rabbit anti-MuSK 1:500, Abcam; rabbit anti-ADRB2 1:200; Santa Cruz Biotechnology; mouse anti-α-dystroglycan 1:100; Santa Cruz Biotechnology) in blocking buffer for 2 hours at room temperature. Sections were washed and incubated in Alexa Fluor® 488 α-BTX (1:250), and secondary antibodies Alexa Fluor® 594 goat anti-rabbit (1:250) for 1 hour at room temperature. Sections were then washed and mounted using Vectashield mounting medium.

Quantification of MuSK, agrin and ADBBR2 staining intensity

Quantification of relative staining intensities was performed as described previously (36,81). Transverse sections of gastrocnemius (MuSK and ADBR2 staining) or whole mounts of lumbrical muscles (agrin staining) were visualised using a Nikon A1R laser scanning confocal microscope. Laser power and parameter settings were kept constant and Z-stack images (1-µm intervals) were acquired with x63 oil immersion objective and processed using NIS-elements AR 4.20.02 software. Confocal micrographs of control and experimental mice were collected in the same session. Quantification of relative area and fluorescence intensity of α-BGT to MuSK, agrin or ADBR2 was performed using ImageJ software. The perimeters of clusters were delimited on the α-BGT channel and the area and fluorescence intensity measured. This selection was restored in the Alexa Fluor® 594 channel and the area and fluorescence intensity of MuSK, agrin or ADBR2 was measured. The background intensity in each channel was subtracted from the pixel intensity of the protein of interest, and the background-corrected intensity for each channel were divided to give a MuSK/agrin/ADBR2-to-α-BGT fluorescence intensity ratio. For MuSK and ADBR2 fluorescence intensity, gastrocnemius muscles from six mice per group were imaged; ≥40 NMJs were analysed per muscle. For agrin fluorescence intensity, lumbrical muscles from six mice per group were imaged and 20 NMJs were analysed per muscle. All imaged AChR clusters were measured.

Fibre type identification

Transverse 10 µm sections of soleus and gastrocnemius muscles were cut using a cryostat and labelled for Myosin Heavy Chains (MHC) MHCI, MHCIIa, and MHCIIb and MHCIIx. Sections were blocked (10% normal goat serum in PBS) for 1 hour at room temperature and then incubated with primary antibodies: rabbit polyclonal IgG anti-laminin (Sigma 1:750), MHCI (BA-F8 Mouse monoclonal IgG2b) 1:25, MHCIIa (Sc71, Mouse monoclonal IgG1), MHCIIb (BF-F3 Mouse monoclonal IgM) 1:200 and MHCIIx (6H1 Mouse monoclonal IgM) 1:25, all DSHB, for 1 hour at room temperature. Sections were then washed in PBS and incubated in secondary antibodies for 1 hour, washed and mounted using Vectashield mounting medium. Images were captured using a Zeiss Axio Imager fluorescent microscope with Zen software and analysed using ImageJ software. In each section, every adjacent field was examined moving from left to right in a systematic manner until the required number was reached. Cross-sectional areas and fibre type proportions were measured in 500 fibres from six non-over-lapping fields at x40 view from six muscles per group.

Transmission electron microscopy

Fresh tissue samples of intercostal muscles were fixed in 2% glutaraldehyde (TAAB Lab), osmicated in 1% osmium tetroxide (Agar Scientific), dehydrated and embedded in epoxy resin (Epoxy embedding resin kit, TAAB Lab). Semi-thin survey sections of 0.5 µm were cut and stained with 1% toluidine blue in 1% borax. Ultrathin sections (70 nm approximately) were then cut and stained with 2% aqueous Uranyl Acetate and Lead Citrate (Leica). The grids were examined on a Hitachi HT7800 transmission electron microscope using an Emsis Xarosa camera with Radius software. Four intercostal muscles from three animals of each group (salbutamol treated ColQ$^{-/-}$, water treated ColQ$^{-/-}$ or WT) were subjected to EM analysis. Quantitative analysis was done with 15 or more electron micrographs analysed by ImageJ. At each distinct region of postsynaptic folding the following features were measured, as described by Slater et al (43): nerve terminal area (total area of axon terminal); presynaptic length (total length of the nerve terminal in direct contact with the muscle fibre); postsynaptic area (total area containing postsynaptic folds); postsynaptic length (total surface length of subneural apparatus measured along the tops of the folds); fold length (total length of postsynaptic membrane including folds); fold number (the number of distinct postsynaptic folds). These measurements were used to calculate the following derived variables: (i) Occupancy (presynaptic length/postsynaptic length); (ii) fold index (fold length/postsynaptic length); (iii) fold density (fold length/postsynaptic area).

Statistical analysis

Data are expressed as means ± S.D. unless otherwise stated. Statistical analyses were performed using GraphPad Prism Version 7 (GraphPad, San Diego, CA, USA) by pair-wise comparisons between 2 conditions with unpaired Student's *t* tests or Mann-Whitney *U* tests. We confirmed normal distributions of data before performing parametric tests using the D'Agostino Pearson omnibus normality test. $P < 0.05$ denoted significance. Datasets were tested for outliers using the ROUT method (robust regression and outlier removal; Q = 1%). None of the outliers affected statistical significance and all were included in analysis. Images were analysed in a blinded fashion by the same experimenter.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its Supplementary material. Inquiries for additional data are available from the corresponding author, upon reasonable request.

Supplementary Material

Supplementary Material is available at *HMG* online.

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ORIGINAL COMMUNICATION

Congenital myasthenic syndrome with episodic apnoea: clinical, neurophysiological and genetic features in the long-term follow-up of 19 patients

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Abstract

Background Congenital myasthenic syndrome with episodic apnoea (CMS-EA) is a rare but potentially treatable cause of apparent life-threatening events in infancy. The underlying mechanisms for sudden and recurrent episodesof respiratory arrest in these patients are unclear. Whilst CMS-EA is most commonly caused by mutations in *CHAT*, the list of associated genotypes is expanding.

Methods We reviewed clinical information from 19 patients with CMS-EA, including patients with mutations in *CHAT*, *SLC5A7* and *RAPSN*, and patients lacking a genetic diagnosis.

Results Lack of genetic diagnosis was more common in CMS-EA than in CMS without EA (56% $n = 18$, compared to 7% $n = 97$). Most patients manifested intermittent apnoea in the first 4 months of life (74%, $n = 14$). A degree of clinical improvement with medication was observed in most patients (74%, $n = 14$), but the majority of cases also showed a tendency towards complete remission of apnoeic events with age (mean age of resolution 2 years 5 months). Signs of impaired neuromuscular transmission were detected on neurophysiology studies in 79% ($n = 15$) of cases, but in six cases, this was only apparent following specific neurophysiological testing protocols (prolonged high-frequency stimulation).

Conclusions A relatively large proportion of CMS-EA remains genetically undiagnosed, which suggests the existence of novel causative CMS genes which remain uncharacterised. In light of the potential for recurrent life-threatening apnoeas in early life and the positive response to therapy, early diagnostic consideration of CMS-EA is critical, but without specific neurophysiology tests, it may go overlooked.

Keywords Congenital myasthenic syndrome · Neuromuscular disease · Neurophysiology · Neuromuscular junction

Background

Congenital myasthenic syndromes (CMS) are a group of disorders caused by mutations in genes encoding proteins responsible for the function and integrity of the neuromuscular junction (NMJ), resulting in impairment of the safety margin necessary for reliable neuromuscular transmission. The first identified mutations were in genes encoding subunits of the acetylcholine receptors (AChRs), and these remain the most common subtypes of CMS worldwide today [1]. However, in recent years the discovery of CMS-related genes has accelerated, and to date over 30 genes have been implicated (Fig. 1).

Although the clinical spectrum is increasingly diverse, CMS are characterised by fatigable weakness typically of early onset, a positive family history, and abnormal neurophysiology tests, namely repetitive nerve stimulation (RNS) or single-fibre EMG (SFEMG) [1]. In RNS, supramaximal electrical stimulation is delivered to a motor nerve via surface electrodes. In normal muscle, the resulting response (known as the compound muscle action potential or CMAP)

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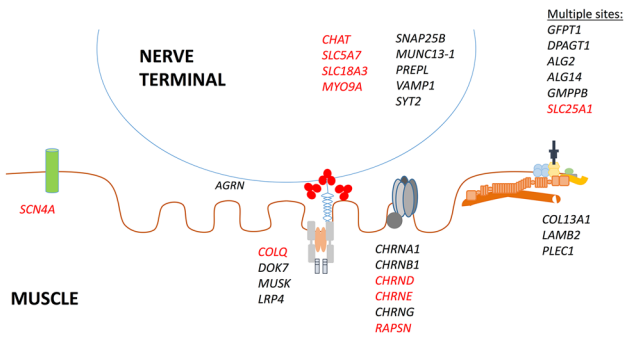

Fig. 1 Heterogeneity of genetic defects in CMS: Mutations are described in genes encoding pre-synaptic, synaptic and post-synaptic proteins, proteins of the extracellular matrix and dystrophin-associated glycoprotein complex and in ubiquitously expressed proteins involved in glycosylation (*GFPT1, DPAGT1, ALG2, ALG14, GHMPPB*) and mitochondrial function (*SLC25A1*) which may act at multiple sites. Genes in which mutations have been previously described in patients with EA are highlighted in red

amplitude remains constant over a wide range of frequencies, since the safety margin for neuromuscular transmission is large. In patients with abnormalities of neuromuscular transmission however, the CMAP amplitude can vary with repetitive stimulation. In pre-synaptic disorders involving defects of neurotransmitter release, the baseline CMAP amplitude is characteristically small, with high-frequency nerve stimulation causing an incremental CMAP response as a result of calcium build-up in the pre-motor terminal. In contrast, in post-synaptic disorders the slight reduction in the release of the neurotransmitter acetylcholine (ACh) caused by low-frequency stimulation can cause the end-plate potential to fall below the threshold required to generate a muscle action potential in a proportion of the muscle fibres. This results in decrement of the CMAP amplitude. However, in many cases, whether the disorder is pre- or post-synaptic cannot be effectively differentiated using neurophysiological testing alone.

In CMS due to mutations in the gene encoding the pre-synaptic choline acetyltransferase (*CHAT*), the deficit is not in calcium-triggered ACh release, but in re-synthesis of ACh following re-uptake by the nerve terminal [2, 3]. Since the terminal contains a large reserve pool of ACh vesicles, a decremental response on RNS is only apparent once this pool has been exhausted. Hence, low-frequency stimulation typically produces no decrement, with the abnormality only becoming apparent following prolonged high-frequency nerve stimulation (e.g. 10 Hz for 5 min).

SFEMG determines the variability in the latency of neuromuscular transmission for individual muscle fibres within the same motor unit, or "jitter". Increased jitter is more sensitive for detecting impaired neuromuscular transmission than RNS, but is less specific since the findings are essentially identical for both pre- and post-synaptic disorders and hence must be interpreted along with clinical symptoms and signs.

CMS may be particularly difficult to diagnose in neonates, in whom non-specific features such as generalised hypotonia, arthrogryposis and poor suck or cry may be the only clinical signs. In this age group, certain subsets of CMS may be associated with life-threatening episodic apnoea (CMS-EA); a rare, but potentially treatable cause of apparent life-threatening events (ALTEs). Whilst CMS-EA was initially described in association with mutations in *CHAT* [2], the genetic basis has expanded, and several more recently described CMS genes have been shown to be associated with EA (Table 1). Genetic diagnosis for these conditions is all the more imperative given that effective treatments may prevent these life-threatening crises, but treatment response varies depending on the genetic subtype. The underlying mechanism of EA in disorders of neuromuscular transmission is unknown. We reviewed the clinical course of 19 patients with CMS-EA, to demonstrate potential diagnostic pitfalls and to assess long-term prognosis.

Methods

Clinical information was reviewed from 34 patients who currently attend the CMS clinic at the John Walton Muscular Dystrophy Research Centre, Newcastle Upon Tyne, United Kingdom, and 929 patients from around the world who were referred to our laboratory for genetic testing between 1997 and 2011 in the context of a clinical suspicion of CMS following review by experienced neuromuscular clinicians [4]. Patients had convincing evidence of CMS on the basis of clinical features, neurophysiological studies, laboratory investigations (including measurement of AChR antibodies and serum creatine kinase) and, in many cases, a prior muscle biopsy. EA was noted as being present where the clinician had recorded recurrent periods of respiratory arrest during the disease course; patients described as having respiratory insufficiency (causing inadequate oxygenation) but lacking clear description of recurrent apnoeic events (with cessation of respiration) were not included. CMS-EA was clearly described in 32 patients; however, detailed clinical information and follow-up were available for 19 CMS-EA patients. For these cases, clinical, genetic and neurophysiological data were retrospectively reviewed. Genomic DNA samples or EDTA blood were provided to our laboratory from neuromuscular and neurology centres worldwide. Sanger-based mutation screening was carried out on a gene-after-gene basis according to phenotype. In one case, which has been previously described, the causative gene (*MYO9A*) was identified following whole exome analysis [5]. Informed

Table 1 CMS genes associated with EA

Gene	Protein and function	Clinical features
CHAT	Choline acetyltransferase (ChAT); re-synthesis of acetylcholine (ACh) from choline and acetyl-CoA in pre-synaptic nerve terminal [2]	Can exhibit striking clinical variability both between and within families. Positive response to AChEIs is seen in almost all cases [3, 22]
RAPSN	Rapsyn; post-synaptic scaffolding protein, interacts with AChRs to induce clustering [23]	Two main phenotypes: late-onset with fatigable limb weakness, early onset characterised by arthrogryposis, high-arched palate, and facial, cervical and bulbar weakness [24–26]
CHRNE (fast channel)	Epsilon subunit of AChR, altered kinetic properties following binding of ACh to receptor [27, 28]	Severe weakness with crises [29]
SLC5A7	High-affinity choline transporter 1, resynthesises ACh in the pre-synaptic nerve terminal [30]	Phenotypes range from severe form with arthrogryposis, hypotonia and early lethality, to neonatal onset CMS with prominent EA [20]
SLC18A3	Vesicular acetylcholine transporter (VAChT), uptake of ACh into pre-synaptic vesicles [20]	Ptosis, ophthalmoplegia and fatigability. Deterioration of symptoms in cold temperature described in one case [20]
COLQ	ColQ, collagenic tail of AChE which anchors AChE in the post-synaptic membrane [31, 32]	Broad phenotype, from adult onset limb girdle CMS to early onset severe and progressive forms. Slowing of the pupillary light reflex (25% of cases). Worsening with AChEI therapy [31, 33]
CHRND	Delta subunit of AChR	Phenotype overlapping with rapsyn-CMS [34–36]
SCN4A	Voltage gated sodium channel (Nav1.4), influx of sodium ions into post-synaptic membrane and generation of muscle action potential [37, 38]	Relatively severe limb weakness, ophthalmoplegia and ptosis. Apnoeic attacks which persisted from infancy into early adulthood are described [38]
MYO9A	Unconventional myosin Myo9A, presumed pre-synaptic function [5]	Identified in two families, neonatal onset EA which responded dramatically to pyridostigmine and 3,4-DAP is described [5]

consent was obtained from all participants by local institutions. All genetically undiagnosed patients had been screened for mutations in *CHAT, RAPSN, CHRNE, COLQ, DOK7, CHRNA1, CHRND, CHRNB1, SLC5A7* and *SLC18A3*. Neurophysiological assessment was performed at local institutions according to protocols described previously [6, 7]. Decrement was defined as a decrease in CMAP amplitude of 10% or more between the first and fourth CMAPs.

Results

Episodic apnoea was described in 3% ($n = 32/963$) of CMS cases. Of the CMS-EA cases, 44% ($n = 14/32$) were genetically diagnosed, compared with 93% ($n = 866/963$) of all CMS cases. The most common causative mutations were in the *CHAT* gene (16% of CMS-EA cases), with mutations in *COLQ* (9%), *RAPSN* (6%), *SLC5A7* (6%), *MYO9A* (3%) and *CHRNE* (3%) being causative for the remainder of the cases with genetic diagnoses (Fig. 2).

In 13 cases, there was insufficient information on long-term follow-up and these were excluded from further analysis. Clinical features, neurophysiology and management are described, therefore, for the remaining 19 cases (Table 2).

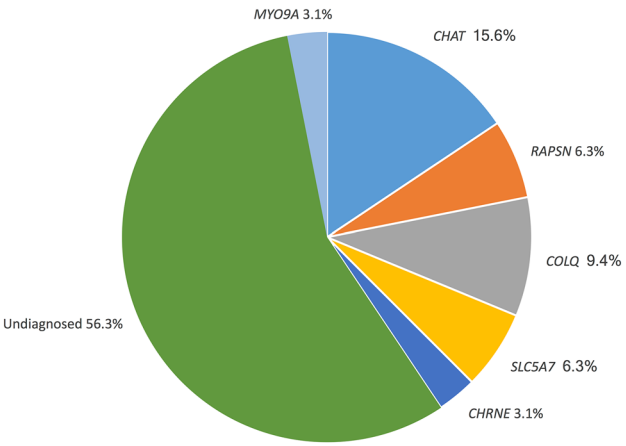

Fig. 2 Proportion of CMS-EA subtypes in our patient cohort (n=32)

Clinical features

All cases were proband, apart from cases 14 and 15 who are siblings. There was no family history of a neuromuscular disorder in any case, and consanguinity was reported in only two families. The disease manifested at birth in 84% ($n = 16$) of cases, with the remaining three cases presenting within the first 2 months of life. Signs present at birth were hypotonia (16 cases) and arthrogryposis (4 cases).

Table 2 Clinical and neurophysiological features in 19 cases of CMS-EA

	Current Age, years	Causative gene; mutation	Cranial muscles	Weakness (distrib); Fatigability	Additional features	Cognition	Neurophysiology (age at assessment)	Treatment and response
Case 1	14	*CHAT;* 2081C > G (S694C) ex 18, 1061C > T (T354M) ex 10	Pto, bulb	Yes (prox); O/E		Severely impaired	RNS—no decrement on 10 Hz stimulation (5 months)	AChEI—transient improvement
Case 2	14	*CHAT;* 2081C > G (S694C) ex 18, 1061C > T (T354 M) ex 10	Pto	Yes (prox); O/E		Normal	RNS—no decrement on 3 Hz stimulation. **20% decrement on prolonged high-frequency stimulation of facial and distal muscles** (7 months)	AChEI—good response
Case 3	8	*CHAT;* 1408C > T (R470X) ex 11, 1730T > G (F580C) ex 13	Ophth, pto	Yes (glob); O/E	AMC	Mildly impaired	RNS—borderline decrement on 3 Hz stimulation, **90% decrement on prolonged high-frequency stimulation**. SFEMG - Increased jitter (3 months)	AChEI—good response. 3,4-DAP—good response
Case 4	3	*RAPSN*; 264C > A (N88 K) ex 2, 1169G > A (C390Y) ex 8	Pto, bulb	Yes (prox, WE, FE); Hx	AMC	Mildly impaired	RNS—20% decrement on 3 Hz stimulation (7 months)	AChEI—good response
Case 5	11	Unknown	Ophth, pto, bulb	Yes (prox, NE, FE); Hx		Normal	RNS—10% decrement on 3 Hz stimulation (10 months)	AChEI—no response. Salb—good response
Case 6	31	Unknown	Ophth, pto, bulb	Yes (glob); Hx		Mildly impaired	RNS—10% decrement on 3 Hz stimulation (11 years)	AChEI—good response. 3,4—DAP—no response
Case 7	16	Unknown	Ophth, pto, bulb	Yes (glob); No	AMC, hip dysplasia, scoliosis, high-arched palate	Mildly impaired	RNS—10% decrement on 3 Hz stimulation (2 years)	AChEI—no response. Salb—no response
Case 8	33	Unknown	No	Yes (dist); Hx		Normal	RNS—20–30% decrement on 3 Hz stimulation (3 years)	None tried
Case 9	14	*SLC5A7;* 331T > C (Y111H), 1252T > G (F418 V)	Ophth, pto, bulb	Yes (prox); O/E		Mildly impaired	RNS—no decrement on 3 Hz stimulation (3 years)	AChEI—no response. Salb—no response
Case 10	6	*SLC5A7;* 524A > G (Y175C), c.1030G > C (V344L)	Ophth, pto, bulb	Yes (glob); No		Severely impaired	RNS—70% decrement on 3 Hz stimulation (14 months)	AChEI—good response. Salb—no response

Table 2 (continued)

	Current Age, years	Causative gene; mutation	Cranial muscles	Weakness (distrib); Fatigability	Additional features	Cognition	Neurophysiology (age at assessment)	Treatment and response
Case 11	1	Unknown	Ophth, pto, bulb	Yes (glob); Hx		Normal	RNS—no decrement on 3 Hz stimulation (5 months)	AChEI—no response
Case 12	7	Unknown	Pto, bulb	Yes (prox); Hx		Normal	RNS—no decrement on 3 Hz stimulation (5 years)	AChEI—good response. Salb—transient improvement
Case 13	13	Unknown	Ophth, pto, bulb	Yes (prox); Hx	AMC, hip dysplasia, high-arched palate	Normal	RNS—no decrement on 3 Hz stimulation (4 years). Repeat RNS—**significant increment in APB (25–34%) and ADM (29–41%) at 30 Hz prolonged high-frequency stimulation** (7 years)	AChEI—good response
Case 14	1	Unknown	Pto, bulb	Yes (prox); Hx		Normal	RNS—decrement at 0.5 Hz stimulation, SFEMG—marked jitter and blocking (1 year)	AChEI—good response
Case 15	6	Unknown	Pto, bulb	Yes (prox); O/E		Normal	RNS—no decrement on 3 Hz stimulation, **prolonged high-frequency stimulation revealed decrement of 50% with partial recovery** (10 weeks)	AChEI—good response
Case 16	9	Unknown	Pto, bulb	Yes (prox); O/E		Normal	RNS—no decrement at 3 Hz, **71–84% decrement on prolonged high-frequency stimulation** with partial recovery (5 years)	AChEI—good response
Case 17	6	*CHRNE;* 43T > C (Y15H) ex 2, 113C > A (T38 K) ex 2	Ophth, pto, bulb	Yes (prox); O/E		Normal	RNS—decrement at 3 Hz in ADM, APB and ADM (8 months)	AChEI—transient improvement. Salb—good response.
Case 18	12	*CHAT;* 1642C > T (R548X) ex 15; 1669G > A (A557T) ex 15	Ophth, Pto, bulb	Yes (prox, NE); O/E		Mildly impaired	RNS—decrement at 3 Hz stimulation. SFEMG—borderline jitter (50 µs MCD) in deltoid (1 year)	AChEI—good response. Salb—good response.

Table 2 (continued)

	Current Age, years	Causative gene; mutation	Cranial muscles	Weakness (distrib); Fatigability	Additional features	Cognition	Neurophysiology (age at assessment)	Treatment and response
Case 19	4	Unknown	Ophth, pto, bulb	Yes (axial); O/E	High-arched palate	Mildly impaired	RNS—no decrement at 3 Hz stimulation, **38% decrement on prolonged stimulation** (5 months). Repeat RNS—no decrement at 3 Hz, **48% decrement on prolonged stimulation**; SFEMG—jitter and blocking (4 years)	AChEI—good response

All genetically undiagnosed patients were screened for mutations in *CHAT*, *RAPSN*, *CHRNE*, *COLQ*, *DOK7*, *CHRNA1*, *CHRND*, *CHRNB1*, *SLC5A7* and *SLC18A3*

3,4-DAP 3,4-diaminopyridine, *AChEI* acetylcholinesterase inhibitor, *AMC* arthrogryposis multiplex congenital, *bulb* bulbar weakness, *dist* distal, *FE* finger extensor weakness, *glob* global, *Hx* fatigability reported on clinical history, *NE* neck extensor weakness, *O/E* fatigable on examination, *ophth* ophthalmoplegia, *prox* proximal, *pto* ptosis, *RNS* repetitive nerve stimulation, *Salb* salbutamol, *SFEMG* single-fibre electromyography

The cases which demonstrated decrement on RNS only after prolonged high frequency stimulation are highlighted in bold

Antenatal complications were recognised in 42% ($n = 8$) of patients, and included reduced foetal movements and/or polyhydramnios.

Age of onset of EA was variable. Most patients had their first apnoea in the hours following birth ($n = 5$, 26%) or in the first 4 weeks of life ($n = 5$, 26%). For four cases (21%), the first apnoeic event occurred between the age of 1–4 months, and four cases developed EA between 4 and 12 months of age. In one case, EA did not occur until the age of 18 months.

Cognitive development was abnormal in nine cases (47%); in three cases, this was severe and considered to be secondary to hypoxic brain damage. Brain MRI was available for ten patients, and described as normal in seven, with two cases showing features in-keeping with hypoxic ischaemic encephalopathy, and one case (with *CHAT*-CMS) showing hypoplasia of the cerebellar vermis.

Median diagnostic delay from first symptoms to the clinical diagnosis of CMS (prior to genetic confirmation) was 8 months (range 1–96 months). Differential diagnoses included laryngomalacia, epilepsy, cardiac conduction defects, congenital hypothyroidism, Prader–Willi syndrome and spinal muscular atrophy.

Apnoeic events and respiratory function

Infections, including respiratory tract infections, were the most common precipitating factor for apnoeas, reported in 74% ($n = 14$) of patients (Table 3). Apnoeas were also reported during feeding, stress, crying, increased activity and increased environmental temperature. Frequency of apnoeas varied significantly, independent of genotype, from approximately one episode per month, to very frequent episodes occurring up to 40 times per day.

During apnoeic episodes, myasthenic features typically worsened, with worsening of hypotonia, bulbar weakness and ptosis reported in 14, 6 and 4 cases, respectively. Approximate duration of events ranged from 30 s to over 30 min (persisting until the patient was intubated and ventilated, and resulting in permanent brain damage), but mean duration of a typical event was 2 min.

There was significant morbidity and mortality in the cohort, with two patients having died following prolonged apnoeas, aged 11 months (genetically undiagnosed) and 3 years (with *RAPSN* mutations), respectively, and a further case with severe hypoxic brain injury being permanently ventilated (*SLC5A7* mutations). However, overall progressive improvement and tendency towards resolution of apnoeic episodes was reported in the majority of cases (Fig. 3). For 11 cases there was complete remission of apnoeas (mean age of resolution 2 and 5 months). A further five cases showed tendency towards remission but still experienced apnoeas during infections.

There was no genotype–phenotype correlation between cases who had remission of EA and those who still experienced apnoeas.

Neurophysiology

All patients had neurophysiological assessment; 4 cases had RNS and SFEMG, and 15 cases had been investigated using RNS alone. Abnormal RNS was seen in 15/19 cases (79%); in four cases, in whom only RNS was performed, no decrement was detected. In 5 of the 15 cases with abnormal RNS, stimulation at 3 Hz was borderline or negative, but prolonged high-frequency RNS (10 Hz for 5 min) revealed marked decrement. In a further patient (genetically undiagnosed) increment was detected on prolonged stimulation. All cases who had SFEMG in addition to RNS demonstrated increased jitter.

Management

18 cases had been treated with acetylcholinesterase inhibitors (AChEIs) at some point. Of these, 67% ($n = 12$) had a sustained clinical improvement, which included all patients with mutations in *RAPSN* and *CHAT*, one patient with *SLC5A7* mutations and seven patients who were genetically undiagnosed. Transient improvement but subsequent deterioration following commencement of AChEIs was seen in two patients (both genetically undiagnosed). AChEIs had no effect in four cases (three genetically undiagnosed, and one *SLC5A7*-CMS). The β_2-agonist salbutamol resulted in clinical improvement in three cases (genetically undiagnosed, fast channel syndrome and *CHAT*-CMS), transient improvement in one case (undiagnosed) and no response in the remaining three cases (one undiagnosed, and two with *SLC5A7*-CMS). 3,4-Diaminopyridine (3,4-DAP) was used as an adjunctive therapy in two cases, resulting in improvement in one (with *CHAT*-CMS) and no response in the other (genetically undiagnosed). All six cases in whom abnormal RNS had only been detected following prolonged high-frequency stimulation had clinical benefit from AChEI therapy.

Most patients ($n = 14$, 74%) had normal respiratory function between apnoeic events. Three cases required nocturnal non-invasive ventilation (one with *CHAT*-CMS and two genetically undiagnosed cases), and one was permanently ventilated via tracheostomy. Hospital attendances were common, with 41% ($n = 8$) of cases requiring frequent (> 1 per year) admissions. Fourteen patients had required ventilation during periods of respiratory crisis (11 invasive and 3 non-invasive ventilation).

Table 3 Apnoeic events and respiratory function in our patient cohort

	Precipitating events	Associated symptoms during apnoea	Recurrent LRTI	Respiratory function between episodes
Case 1	Feeding, infection, stress	Hypotonia, bulbar weakness, ptosis	Yes	Normal
Case 2	Feeding, infection, stress	Hypotonia	Previously, now resolved	Normal
Case 3	Infection	Hypotonia	Previously, now resolved	Normal
Case 4	Infection, feeding	Hypotonia	Yes	Normal
Case 5	Infection, during sleep	Hypotonia, bulbar weakness	Yes	Normal
Case 6	Stress, infection, higher temperature	Ptosis, bulbar weakness, hypotonia	Previously, now resolved	Normal
Case 7	Crying	Hypotonia	No	Normal
Case 8	Infection	None noted	Previously, now resolved	Normal
Case 9	Feeding, infection	Ptosis, bulbar weakness, hypotonia	No	Normal
Case 10	Infection	Hypotonia	Yes	Permanently ventilated
Case 11	Infection, feeding, crying	None noted	Yes	Normal
Case 12	Feeding, infection	Hypotonia, facial weakness	Yes	Impaired
Case 13	Feeding	No	Yes	Normal
Case 14	Feeding	Hypotonia, ptosis, cyanosis	Yes	Not assessed
Case 15	Infection, increased activity, sleep	Hypotonia	Yes	Normal
Case 16	Infection, increased activity, sleep	Hypotonia, bulbar weakness	Yes	Normal
Case 17	Feeding	Hypotonia	No	Normal
Case 18	Feeding, infection, during sleep	Hypotonia, cyanosis	Yes	Impaired
Case 19	Infection, feeding	Hypotonia, cyanosis	Yes	Impaired

LRTI lower respiratory tract infection

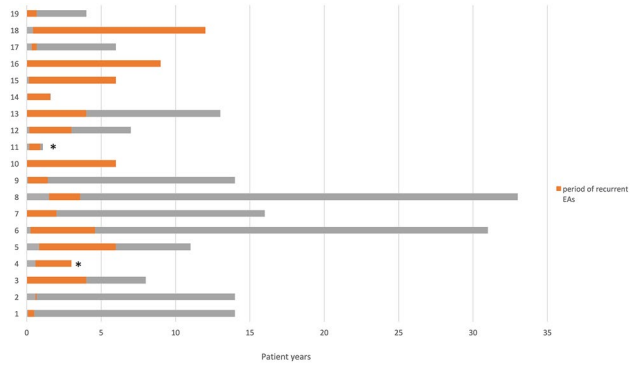

Fig. 3 Resolution of apnoeic events over time: For the majority of cases, the period of recurrent EAs (orange) during a patient's life span (gray) began in the first months of life and resolved in early childhood. Cases marked * are deceased

Discussion

Apparent life-threatening events (ALTEs), defined as episodes characterised by some combination of apnoea, skin colour change, change in muscle tone and choking or gagging [8], have an extremely broad differential diagnosis. Causes include epilepsy, cardiac arrhythmia or structural cardiac defects, gastro-oesophageal reflux disease, respiratory tract infections and upper airway obstruction [9–12]. ALTEs are most common in the first 10 weeks of life, with cyanosis and apnoea often being the most prominent or only symptoms [11]. CMS is rarely considered as a possible differential diagnosis for ALTEs, thus many patients undergo extensive investigation before the possibility is considered. In our cohort, average diagnostic delay was substantial at 8 months. Worsening of myasthenic symptoms during apnoeas was frequently observed, and these features may provide a diagnostic clue. In practice, however, many patients may be admitted directly to intensive care units and may not be examined by a paediatric neurologist or neuromuscular specialist during these crises.

In early infancy, abnormal neurophysiology tests may be the only indication of a NMJ disorder. However, we observed in this cohort that without specific neurophysiological assessment, a pre-synaptic abnormality may go overlooked. In 32% ($n = 6$) of cases, no abnormality was observed at low-frequency RNS, with decrement or increment only becoming apparent following prolonged high-frequency stimulation. In two of these cases, the genetic diagnosis was of ChAT deficiency, in which re-synthesis of ACh is known to be impaired. In the other four cases, the genetic diagnosis was unknown, but it may be that these also affect proteins involved in this pathway. Failure to perform this additional test could result in the diagnosis of CMS-EA being discounted. Furthermore, all of these cases showed a positive response to AChEI therapy. Such prolonged studies can be uncomfortable for the patient, and may require sedation. Nevertheless, given the risk of fatal apnoeas if the diagnosis is missed, and the potential for clinical improvement with AChEIs, these prolonged studies should be performed if deficiency of ACh re-synthesis is suspected.

The underlying mechanism(s) giving rise to these sudden and recurrent apnoeas is yet to be characterised. Respiratory control mechanisms respond to input from neural and chemical receptors, which are integrated by the respiratory centres in the medulla and pons. These subsequently provide neuronal drive to respiratory muscles, maintaining upper airway patency and determining the level of ventilation. Recurrent apnoea could be due to an abnormality at any point along this axis. The majority of mutations causing CMS-EA lead to pre-synaptic defects (Fig. 1), which have important functions for both central and neuromuscular synaptic function. This, along with the tendency for patients to have normal respiratory function between apnoeas, could point to a centrally mediated mechanism. In addition, the proportion of patients with impaired cognitive function in our cohort (47%) was higher than expected and is not something that has been previously well-characterised in disorders of the NMJ. Any impairment of higher function in CMS-EA patients is often explained by hypoxic brain damage due to repeated respiratory failure, although the possibility of impaired CNS maturation due to reduced neurotransmitter in central cholinergic neurons has not been fully explored. The most common CMS-EA gene, *CHAT*, has important functions in both central and peripheral synapses, and deficiency of ChAT has been reported in Alzheimer's disease, idiopathic Parkinson's disease, Huntington's disease and schizophrenia [13–16]. Furthermore, in cases of sudden infant death syndrome, decreased activity of ChAT has been demonstrated in the CNS [17, 18]. Cognitive and behavioural have also been described in the recently identified CMS subtype due to mutations in *SLC5A7*, which encodes the high-affinity choline transporter 1, necessary for uptake of choline from the synaptic space for the synthesis of ACh at central and peripheral cholinergic synapses [19]. A further CMS-EA gene involved in ACh release, *SLC18A3*, encodes the vesicular acetylcholine transporter (VAChT), which mediates the vesicular storage of ACh in the central and peripheral nervous system. *SLC18A3* mutations are associated with a severe CMS phenotype and learning difficulties are also described [20, 21]. The CMS-EA genes *SNAP25B*, *VAMP1* and *MUNC13* encode proteins of the SNARE complex. These are essential for calcium-triggered exocytosis at central and neuromuscular synapses; therefore, it is unsurprising that CMS due to mutations in these genes may be associated with epilepsy and impaired brain development [22, 23]. Abnormalities of the CNS have also been reported in CMS due to mutations in the unconventional myosin gene

MYO9A. MYO9A also appears to have pre-synaptic function and localization at the NMJ, although the mechanism for NMJ dysfunction in these patients is yet to be determined [5].

Administration of one or more drugs is indicated once the diagnosis of CMS-EA is established. Several drugs have shown convincingly positive effects in reducing the frequency of apnoeas, including AChEIs, 3,4-DAP and sympathomimetics (ephedrine or salbutamol). Because of the sudden nature of these attacks, parents should be provided with an inflatable rescue bag and fitted mask, trained in CPR and may be provided with a home apnoea monitor. Parents may also be instructed to give additional AChEI doses during infection or other potential precipitating events.

We recognise that this study has several limitations. We include a small number of patients, contributed to by the rarity of the condition. Data were collected retrospectively and over a long period of time (1997–2011) during which the awareness of CMS and availability for genetic testing has increased. However, even in this patient cohort, who continues to be tested for new causative CMS genes as they are discovered, a relatively large proportion (56%) of cases remain genetically undiagnosed. CMS-EA is a challenging diagnosis, with several mimics, and this proportion could suggest an alternative diagnosis other than a neuromuscular transmission defect in these patients. However, it may also reflect further CMS causing genes which have not yet been discovered.

Given the likelihood of recurring episodes, the potential for psychomotor impairment due to secondary hypoxic brain damage, and the positive effect of available treatments, CMS-EA is an important diagnosis not to miss. Prompt diagnosis and initiation of treatment and ventilatory support is imperative in early life, when EA may be frequent, but the majority of cases will exhibit a tendency towards remission. Improved understanding of the mechanism of this condition may provide insights into further causative genes for genetically undiagnosed patients, and lead to improved diagnostic and treatment strategies.

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Compliance with ethical standards

Ethical standards All human studies have been approved by the appropriate ethics committee (Newcastle and North Tyneside 1 Research Ethics Committee) and have, therefore, been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Conflicts of interest The authors declare that they have no conflict of interest.

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Impaired Presynaptic High-Affinity Choline Transporter Causes a Congenital Myasthenic Syndrome with Episodic Apnea

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The neuromuscular junction (NMJ) is one of the best-studied cholinergic synapses. Inherited defects of peripheral neurotransmission result in congenital myasthenic syndromes (CMSs), a clinically and genetically heterogeneous group of rare diseases with fluctuating fatigable muscle weakness as the clinical hallmark. Whole-exome sequencing and Sanger sequencing in six unrelated families identified compound heterozygous and homozygous mutations in *SLC5A7* encoding the presynaptic sodium-dependent high-affinity choline transporter 1 (CHT), which is known to be mutated in one dominant form of distal motor neuronopathy (DHMN7A). We identified 11 recessive mutations in *SLC5A7* that were associated with a spectrum of severe muscle weakness ranging from a lethal antenatal form of arthrogryposis and severe hypotonia to a neonatal form of CMS with episodic apnea and a favorable prognosis when well managed at the clinical level. As expected given the critical role of CHT for multisystemic cholinergic neurotransmission, autonomic dysfunctions were reported in the antenatal form and cognitive impairment was noticed in half of the persons with the neonatal form. The missense mutations induced a near complete loss of function of CHT activity in cell models. At the human NMJ, a delay in synaptic maturation and an altered maintenance were observed in the antenatal and neonatal forms, respectively. Increased synaptic expression of butyrylcholinesterase was also observed, exposing the dysfunction of cholinergic metabolism when CHT is deficient in vivo. This work broadens the clinical spectrum of human diseases resulting from reduced CHT activity and highlights the complexity of cholinergic metabolism at the synapse.

The synapse is a highly specialized structure that is fundamental for the function of the neuron by mediating efficient chemical transmission to its postsynaptic cell. One of the best-studied synapses used as a model is the cholinergic neuromuscular junction (NMJ). Cholinergic transmission is unique among the neurotransmitter systems in that it is rapidly stopped not only by clearance but also by enzymatic cleavage of the neurotransmitter in the synaptic space by cholinesterases to ensure quick successive postsynaptic responses.[1] Toward this aim, the nerve terminal takes up choline from the synaptic space through the presynaptic sodium-dependent high-affinity choline

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Table 1. Clinical Features of the Subjects with a Neonatal Form of CMS-EA due to *SLC5A7* Mutations

	Individual 1 (France)	Individual 4 (Algeria)	Individual 5 (Italy)	Individual 6 (Italy)	Individual 7 (Romania)
Sex; age at onset	male; birth	male; 2 months	female; birth	male; birth	male; birth
Mutations	c.194G>A (p.Gly65Glu), c.313C>T (p.Pro105Ser)	c.143A>G (p.Asp48Gly), c.143A>G (p.Asp48Gly)	c.524A>G (p.Tyr175Cys), c.1030G>C (p.Val344Leu)	c.331T>C (p.Tyr111His), c.1252T>G (p.Phe418Val)	c.872T>C (p.Ile291Thr), c.1336A>G (p.Arg446Gly)
Pregnancy	uneventful	uneventful	uneventful	uneventful	uneventful
First symptoms (age)	sudden apneas (b), hypotonia (b)	long meals (2 months), brief bradypneas (5 months)	respiratory distress (b), sudden apneas (b)	sudden apneas (b), weak cry (b)	sudden apneas (b), hypotonia, cyanosis (b), sucking difficulties (b)
Neonatal period	sudden apneas misdiagnosed as seizures; ventilation till 6 weeks; sucking, swallowing, and chewing difficulties; axial hypotonia	slow feeding; few minutes bradypneas; stridor; swallowing difficulties	frequent apneas requiring ventilation support	frequent apneas; dysphagia; acute ptosis	sudden apneas with cyanosis when crying misdiagnosed as breath holding
Motor delay	yes (walk 22 months)	yes (sit 9 months, walk 20 months)	yes (walking never gained)	yes (walk 17 months)	yes (sit 7 months, walk 18 months)
Symptoms during Course					
FUD	16 years	4 years	5 years	11 years	3 years and 9 months
EA	yes, until starting AChEI at 18 months	2 episodes	yes, till now	no	yes, until starting AChEI at 4 months
CH	yes	no	yes	no	ND
Ventilation; tracheostomy	yes (5 months); yes (from 6 months to 5 years)	no; no	yes; no	no; no	no; no
Bulbar weakness; facial weakness	sucking, swallowing, chewing till 10 months; facial weakness from 18 months till now (mild chewing, articulation)	intermittent stridor, no dysphagia; hypomimia	yes (tube feeding due to swallowing and chewing difficulties); constant facial weakness	yes (nasal voice, occasional dysphagia); no	dysphonia and dysphagia until 2 years; ND
Ptosis; OPH	yes; yes	yes (from 4 months); yes	yes; yes	yes; yes	yes; yes
Limb fatigability; weakness	yes; yes (Gowers till 28 months, run at 6 years), waddling gait	yes; no	yes; severe proximal	yes; yes (lower limb girdle)	yes; fatigability
Axial weakness	yes (neck and spine), improved by AChEI	yes (neck intermittently)	yes	yes (neck, flexion and extension)	yes (normal walk but climbs stairs with one foot at time)
Scoliosis; cont.	mild; no	no; no	no; no	kyphosis; no	no; no
Amyotrophy	no	no	yes	no	no
DMN signs	no	no	no	pes planus	no
CD; behavior	no; normal	no; normal	yes; abnormal	yes; normal	yes; ND
Course Characteristics					
Evolution	marked improvement since AChEI administration at 18 months	improvement since AChEI administration at 18 months	stable with episodic crises, muscle weakness without progression	spontaneous recovery of respiratory crises	improved after increasing AChEI
Fluctuations[a]	yes (few days)	yes (one day)	no	yes (diurnal)	yes (respiratory intercurrence)
Exacerb.; MC	no; no	no; no	no; no	yes; no	yes; ND

(*Continued on next page*)

Table 1. *Continued*

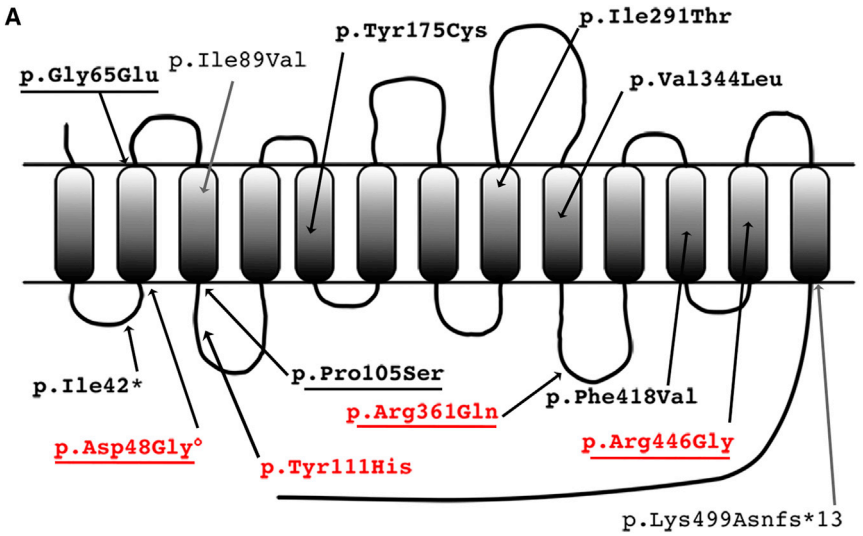
	Individual 1 (France)	Individual 4 (Algeria)	Individual 5 (Italy)	Individual 6 (Italy)	Individual 7 (Romania)
Last Examination					
Age	16 years	4 years	5 years	11 years	3 years and 9 months
PV/NIV; T	no; no	no; no	PV; yes	no; no	no; no
Bulbar; facial	no; yes (minimal)	yes (mild stridor); yes (intermittently)	yes; yes	yes (nasal voice); yes	yes (nasal voice); yes
Ptosis; OPH	yes; yes (fluctuant in severity)	yes; yes (persistent)	yes; yes (persistent)	yes; yes (permanent)	yes; yes (permanent)
Limb weakness; fatigability	no; yes (moderate, lower limbs)	no; yes	generalized and constant severe weakness; no	yes (distal upper and proximal lower); no	yes; yes
Axial weakness	no	yes (neck, intermittent)	yes	yes (neck)	no
EMG (age)	18 months	16 months	14 months	3 years	4 months
Decrement 3 Hz	66%	no, but yes after a pulse of 10 s at 20 Hz	70%	no (no challenging conditions done)	10%–20%
Therapy and effect	AChEI, +++	AChEI, +++	AChEI, +	AChEI,−; salbutamol, −[b]	AChEI, ++

Abbreviations are as follows: b, birth; FUD, follow-up duration; EA, episodic apnea; AChEI, acetylcholinesterase inhibitors; OPH, ophthalmoparesis; CH, chronic hypoventilation; ND, not determined; cont., contractures; DMN, distal motor neuropathy; CD, cognitive deficit; MC, myasthenic crisis; Exacerb., exacerbation; PV, permanent ventilation; NIV, nasal intermittent ventilation; T, tracheostomy.
[a]Except sudden apneas.
[b]Adequate dose and duration of treatment were not optimal.

transporter 1 (CHT) to resynthesize ACh by the action of choline acetyltransferase (ChAT). The high-affinity choline uptake from the synaptic space by this hemicholinium-3 (HC-3)-sensitive transporter CHT represents the limiting step for sustained ACh synthesis and is tightly regulated with ChAT activity.[2]

The NMJ achieves action potential transmission from the motoneuron nerve terminal to the skeletal muscle fiber to control muscle contraction. Defective neurotransmission at the NMJ results in myasthenia, i.e., fluctuating fatigable muscle weakness.[3] The inherited types of myasthenia form the group of congenital myasthenic syndromes (CMSs) characterized by an early age of onset, disease progression, no ACh receptor (AChR) antibodies, and responsiveness to treatments, especially acetylcholinesterase (AChE) inhibitors. Mutations in 23 genes are known to cause CMS. Among them is found *CHAT* (MIM: 118490) that is mutated in a recessive form of CMS with sudden episodes of apnea (CMS-EA [MIM: 254210]).[4,5] On the other hand, one dominant-negative truncating mutation in *SLC5A7* (MIM: 608761) encoding CHT causes a distal hereditary motor neuronopathy, type VIIa (DHMN7A [MIM: 158580]) with progressive distal muscle wasting and vocal cord paresis, questioning the relationship of CHT with CMS.[6] Worldwide, there are still many individuals with a clinical diagnosis of CMS who remain genetically undiagnosed. In this paper, we report loss-of-function mutations of *SLC5A7* in six unrelated families as the underlying cause of a recessive form of CMS that clinically ranges from muscle hypotonia with early neonatal lethality to a neonatal form sharing striking similarities to CMS-EA resulting from *CHAT* mutations.

The six families were part of two distinct European CMS cohorts. Participants gave informed consent through a prospective donor scheme approved by national ethic committees (DC-2012-1535 and AC-2012-1536), and genomic DNA was isolated from blood samples. Medical histories were taken from all participants by neurologists. All parents displayed no peculiar medical history and were healthy at clinical examination. Electroneuromyography (ENMG) and repetitive nerve stimulation (RNS) were carried out under standardized protocols.[7] A detailed clinical description of the two unrelated individuals (1 and 2) who underwent whole-exome sequencing is found in the Supplemental Note, and Table 1 summarizes the clinical features of the five isolated individuals (1, 4–7) with a neonatal form close to CMS-EA.[5] To summarize, brief and recurrent EA occurred neonatally as the inaugural symptom in all subjects with the neonatal form. Other symptoms of CMS were present such as hypotonia, weakness and fatigability, hypomimia, and oculo-bulbar symptoms. A significant decrement in the EMG with RNS was observed in four individuals (1, 4, 5, 7). AChE inhibitors were highly efficient for three subjects (1, 4, and 7) with cessation of the EA and improvement of all myasthenic symptoms. Although individual 6 had no decrement in EMG with RNS, he had mild ptosis worsening at the end of the day, ophtalmoparesis (impaired abduction of left eye and impaired adduction of right eye) with variable strabismus, and striking limb fatigability, all features



B

	48	65	105	111	175	291	344	361	418	446
Hs	GRDIG/VGGGY/AKPMRSKGYVT/TLYTL/ILIGA/AAVMS/FARNI/VIFPQ/FLRIT									
Rt (93)	.....	.....	.....		I....	.C...	.....	.....	I....	.....
Mm (93)	.....	.....	.....		I....	.C...	.....	.....	I....	.....
Dr (74)	.....	.....	.....R....		IF...	V....	.....	.....	I....	I...G
Tm (72)	.....	.....	.....R....		V....	V....	.....	.....	I....	L...S
Ce (50)	..N..	...A.	..K..EE..I.		VF...	A....	.....	..H..	IL...	V..LI

Figure 1. Schematic Representation of CHT and Position of the Variants Linked to the Antenatal Form with Arthrogryposis and to the Neonatal Form of CMS

(A) The substitutions affecting an amino acid residue already described as critical for CHT activity are in red. All amino acid changes result from mutations that were in the heterozygous state except c.143A>G (p.Asp48Gly), which was homozygous (circle). The variants studied at the functional level are underlined. The changes resulting in DHMN7A (p.Lys499Asnfs*13) and linked to ADHD (p.Ile89Val) are also indicated.

(B) The alignment of human CHT with the sequences of five model species (rat, mouse, zebrafish, *Torpedo mamorata*, and *Caenorhabditis elegans* with the percent of amino acid identities in brackets) demonstrates the high conservation of the substituted residues.

consistent with a diagnosis of CMS. The two sibs of family 2 displayed a more severe antenatal phenotype with hydramnios and arthrogryposis of fingers with knees and malformative features in individual 2 (see Supplemental Note). Death occurred at the age of 10 (individual 3) and 15 (individual 2) days.

After exclusion of *CHAT* mutations by Sanger sequencing, whole-exome sequencing was performed in family 1 on blood genomic DNA though enrichment capture using the SureSelect Human All Exon v5 kit (Agilent Technologies) and paired-end massive parallel sequencing in a HiSeq 2000 machine (Illumina) with an average coverage of 60×. Whole-exome sequencing and variant analyses were performed for individual 2 (family 2) as previously described.[8] Two heteroallelic candidate variants in *SLC5A7* were found to be linked with the disease when searching for rare variations (less than 1% of frequency in control databases) with a recessive model of inheritance in the two families (Figure S1). In family 1, two missense variations (c.194G>A [p.Gly65Glu] and c.313C>T [p.Pro105Ser]; GenBank: NM_021815) segregated with the disease. In family 2, the two deceased infants were compound heterozygous for one missense (c.1082G>A [p.Arg361Gln]) and one nonsense (c.123_126del [p.Ile42*]) variation inherited from their parents. No other candidate gene was retained in the two families when recessive inheritance and single-nucleotide variations not reported in polymorphic database were used as filtering parameters.

We then searched for *SCL5A7* mutations by Sanger sequencing of its 10 exons in 95 unrecognized individuals with CMS-EA who did not have mutations in *CHAT*. Four isolated subjects were found to harbor two distinct *SLC5A7* compound heterozygous candidate mutations with a recessive inheritance pattern (individuals 4–7, Table 1). All mutations were inherited except c.331T>C encoding the p.Tyr111His substitution (individual 6), with a maternal mutant allele arising de novo. None of the candidate mutations were reported in control databases (in-house, dbSNP, 1000 Genomes, ExAC), except c.1082G>A (p.Arg361Gln, family 2) and c.872T>C (p.Ile291Thr, individual 7) that were reported in 2 and 7 of 121,350 alleles in the heterozygous state (ExAC), respectively. All missense substitutions were reported as pathogenic by SIFT, PolyPhen-2, and MutationTaster.

The 10 missense mutations were located all along the 580 amino acid residues composing CHT that consists of 13 transmembrane segments (Figure 1A).[9] All substituted for an amino acid residue that is highly conserved during evolution (Figure 1B). Four variations affected amino acids that have been demonstrated to be critical for choline uptake activity (Asp48, Tyr111, Arg361, and Arg446).[2,10] We investigated the pathogenicity of five of these ten missense substitutions that were distributed all along the transporter (c.143A>G [p.Asp48Gly], c.194G>A [p.Gly65Glu], c.313C>T [p.Pro105Ser], c.1082G>A [p.Arg361Gln], and c.1336A>G [p.Arg446Gly]) on CHT activity in vitro to determine their functional effect. We introduced the single-nucleotide changes into the human cDNA encoding CHT (OriGene Technologies) by site-directed mutagenesis (GenScript). The WT and mutant cDNA constructs were transiently transfected into HEK293T cells using Fugene6 reagent (Promega), and membrane expression as well as choline uptake were evaluated. Figure 2A shows typical immunostaining patterns (ApoTome 2 acquisition, Carl Zeiss) obtained on permeabilized cells stained with a monoclonal antibody directed against human CHT (clone 62-2E8,

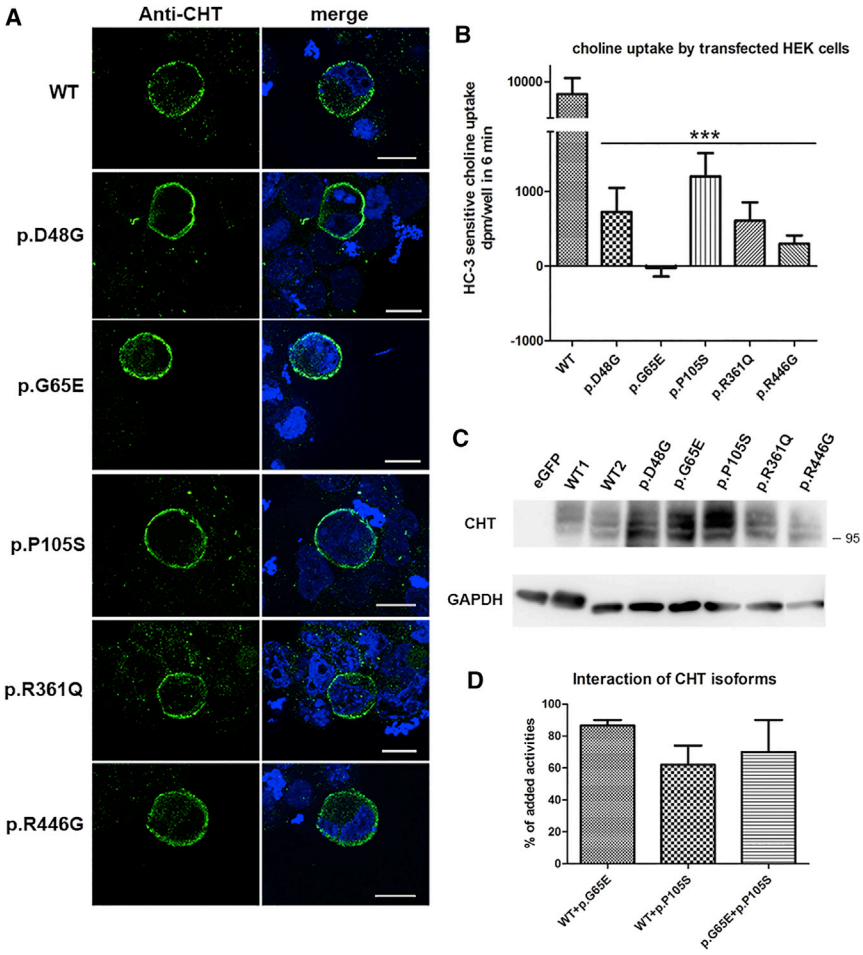


Figure 2. Heterologous Expression and Transporter Activity of CHT in Transfected HEK293T Cells

(A) Immunostaining of heterologous CHT using anti-*SLC5A7* antibodies showed a membranous staining of the wild-type (WT) and the five variant CHT investigated in HEK293T cells transiently transfected with *hSLC5A7* cDNA constructs (c.143A>G [p.Asp48Gly], c.194G>A [p.Gly65Glu], c.313C>T [p.Pro105Ser], c.1082G>A [p.Arg361Gln], and c.1336A>G [p.Arg446 Gly]). Nuclei are stained with DAPI (blue). Scale bars represent 10 μm.

(B) Evaluation of the choline transporter activity sensitive to HC-3, corresponding to the heterologous expression of CHT activity in transiently transfected HEK293T cells. The y axis bar is interrupted for a better visualization of the residual transporter activity of the variant proteins.

(C) Western blot analysis of heterologous CHT in total cell extracts of HEK293 cells transiently transfected with the wild-type or mutant *hSLC5A7* constructs performed in denaturating conditions. Three main bands (95 kDa) were observed using a polyclonal antibody directed against human CHT. Anti-GAPDH antibody was used as loading control. No band specific to CHT was observed in extracts from cells transfected with eGFP alone (eGFP), confirming the specificity of the antibody. The size and amount of the bands specific to heterologous CHT were similar between the wild-type (lines WT1 and 2) and the five variants.

(D) Evaluation of HC-3-sensitive choline transport when coexpressing the wild-type and the c.313C>T (p.Pro105Ser) and c.194G>A (p.Gly65Glu) mutant constructs in transiently transfected HEK293T cells. The activities recorded in cells coexpressing the indicated combinations of cDNA are compared to the summation of the individual activities obtained when the cells were transfected with the wild-type or the mutant constructs.

The results are expressed as mean ± SEM. Statistical significance was calculated by Student's t test with a level of statistical significance set at $p < 0.05$ (***$p < 0.001$).

Sigma-Aldrich). We observed CHT membrane immunolabeling for the five investigated missense mutations, suggesting conserved cell trafficking of the mutated CHT compared to control.

We then determined whether the choline uptake by CHT was altered by the amino acid substitutions. HC-3-sensitive choline transport by transfected cells was evaluated on a period of 6 min using 1 μM ^{14}C-choline (PerkinElmer) subtracting the uptake observed with 1 μM HC-3 (Sigma-Aldrich), since choline uptake by mock-transfected HEK293 cells was not sensitive to this HC-3 concentration.[10] Whole-cell lysates were assayed for accumulated radioactivity by liquid scintillation spectrometry (Packard Tri-carb Liquid Scintillation Counter). Specific CHT-mediated choline uptake was severely impaired for all mutants tested, with residual activity varying between 0% and 10% of control levels (Figure 2B). Although total choline uptake by mock-transfected cells was not saturated in the low micromolar range of choline concentrations, the wild-type CHT-mediated component of choline transport was characterized by a Km of 2.5 μM and a Vmax of 75 pmol/well (0.5 nmol/mg protein) over 6 min. When choline uptake by the p.Asp48Gly and p.Pro105Ser isoforms of CHT was analyzed at this range of concentrations, the low level of uptake did not allow convergence (p.Asp48Gly) or gave a much lower estimate of Vmax at 5 pmol/well, with a Km of 0.8 μM (p.Pro105Ser, data not shown). The other variants were not tested because their residual activity was even less than that of p.Asp48Gly. Western blot analyses of total cell extracts using an affinity purified polyclonal anti-CHT antibody (Abcam) confirmed that the amino acid substitutions did not affect the size of CHT and that the drastic reduction of choline transport activity of mutated CHT was not due to reduced protein level compared to the wild-type CHT (Figure 2C). The size and pattern of the detected bands closely resembled those observed previously for homo-oligomers.[10] CHT functions as a homo-oligomer, and two amino acid substitutions (c.265A>G [p.Ile89Val] and c.1497delG [p.Lys499Asnfs*13]) observed in humans have been reported to exert

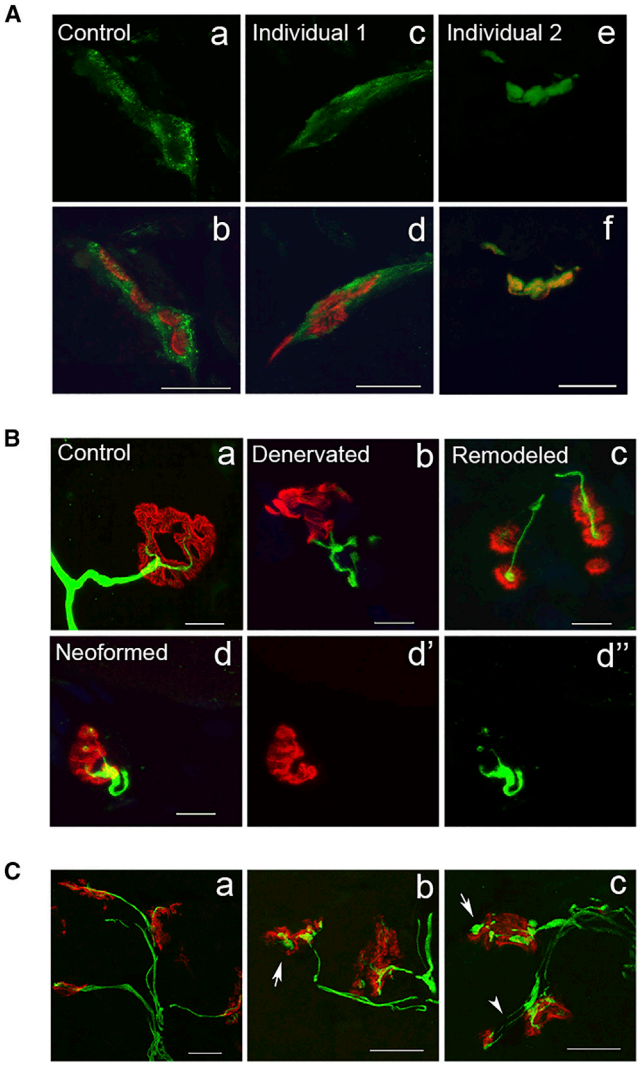


Figure 3. Immunostaining Analyses of NMJs in Muscle Samples from Individuals 1 and 2

(A) CHT immunostaining (green) and post-synaptic nAChR fluorescent staining (red in b, d, f) on transversal muscle sections showed the presence of CHT at NMJs in individuals 1 (c, d) and 2 (e, f) with a staining intensity similar to the adult control (a, b). (B and C) Representative pictures of muscle biopsies stained for the motor axons with an anti-neurofilament (in green) and for post-synaptic nAChR with α-bungarotoxin (in red).

(B) The staining pattern of the two synaptic elements in the young adult individual 1 led us to classify the NMJs in three categories depending upon the innervation status of each NMJ: denervated (b), remodeled (c), or neoformed (d, d′, d″; d is the merged representation of d′ and d″). One NMJ from the adult control (a) with well-defined synaptic gutters responsible for the well-circumscribed post-synaptic nAChR fluorescent staining is shown for comparison.

(C) Three representative images of immature NMJs observed in the autopsy material of the deceased newborn (individual 2) with no well-differentiated pattern for the postsynaptic apparatus (a, b, c), evidence for accumulation of neurofilament staining in nerve terminal (arrow in b and c), and polyinnervation (arrowhead in c). Scale bars represent 10 μm.

a dominant-negative effect on the wild-type CHT.[6,11] We therefore coexpressed the cDNAs containing the c.194G>A (p.Gly65Glu) and the c.313C>T (p.Pro105Ser) mutations—the two compound heterozygous mutations identified in individual 1—with the wild-type cDNA or with each other. The CHT activity of co-transfected cells was less than that calculated from the addition of CHT activities obtained when the cDNA were transfected individually (Figure 2D). However, the impact of the variants on the WT transporter activity was low, concordant with the recessive nature of the tested mutations.

Cholinergic metabolism is critical for NMJ formation, and knock-out *Slc5a7* mice lacking CHT display defective innervation patterns at the NMJ.[12] To determine whether the NMJ structure was impacted when CHT activity is deficient in human, we investigated the NMJs in muscle biopsy samples available for the 16-year-old individual 1 (deltoid muscle) and for the deceased newborn 2 (autopsy material) via standard protocols.[13] CHT was immunostained at the NMJ in the muscle biopsies of the two individuals at a level similar to the control muscle biopsy using a polyclonal antibody (Abcam) as expected from the in vitro analyses of CHT protein levels (Figure 3A). Denervation-reinnervation processes at the NMJs were evident in the muscle sample of individual 1 (Figure 3B and Table S1). Electron microscopy further confirmed the occurrence of a partial denervation-reinnervation process with small nerve terminals and empty synaptic gutters (n = 5; Figure S2A). When present, nerve terminals contained synaptic vesicles that appeared normal in quantity (Figure S2B). Well-defined secondary synaptic folds were observed, suggesting normal formation of the post-synaptic element. Cytoplasmic immunostaining of terminal Schwann cells using S100 antibodies did not detect major abnormalities except for a less well-defined staining pattern compared to control (Figure S3A), which was confirmed at the ultrastructural level (Figure S2A). Autopsy material of newborn 2 showed immature NMJs with thin and unbranched terminal axons contacting undefined subneural folds that sometimes appeared to be polyneuronally innervated (Figure 3C). This is consistent with delayed NMJ development in this newborn, since elimination of polyneuronal innervation should be completed during the 25th week in utero.[14]

To determine whether critical synaptic actors of cholinergic metabolism were impacted by defective CHT activity, we investigated their localization by immunostaining. ChAT (polyclonal antibody, Abcam) and the vesicular acetylcholine transporter VAChT (monoclonal antibody, clone S6-38, Abcam)—which allows the storage of ACh into synaptic vesicles[1]—were present in levels that look similar to controls (Figure S3B). Enzymatic (Koelle-Friedenwald) and fluorescent staining of AChE using fasciculin-2 also appeared similar between case and control subjects (Figure S4A and data not shown). By contrast, the immunostaining of butyrylcholinesterase (BChE, 11D8 clone)[15]—the cholinesterase bound to perisynaptic Schwann cells in mature NMJs[16]—was abnormally strong in the NMJs of both subjects compared to control samples (Figure S4B). These data indicate synaptic remodeling when CHT activity is impaired at the human peripheral cholinergic synapse.

Our data bring the total number of presynaptic forms of CMSs to five and demonstrate that *SLC5A7* is the second most frequent mutated gene linked to a presynaptic form after *CHAT*. The phenotype of the five children presenting with the neonatal form who are reported in this paper is fully reminiscent of recessive CMS-EA due to ChAT deficiency.[5,17] *CHAT* mutations lead to impaired synthesis of ACh by reduced ChAT protein level or kinetic defects depending upon the mutation.[18] The similarity of CMS-EA resulting from *SLC5A7* or *CHAT* loss-of-function mutations is to be expected because both diminish the synthesis of ACh in the nerve terminal, thereby limiting the ability of the cholinergic synapse to continue functioning when the demand of ACh release is high.[2] The association of a lethal antenatal form could be expected because CHT is detected at high levels in E14 rodent spinal cord when NMJ begins to form.[19]

One interesting observation was the strong immunostaining of synaptic BChE observed in the two analyzed muscle biopsy samples. BChE has not been well investigated as an actor in ACh metabolism at the NMJ. This cholinesterase is bound to perisynaptic Schwann cells, and its absence does not cause disease in mice or humans.[16,20–22] The upregulation of BChE does not seem to be a secondary consequence of NMJ remodeling with denervation-reinnervation events because these are frequently observed when analyzing NMJs of individuals with CMS. Conceivably, BChE finely tunes neuromuscular transmission, probably by regulating the binding of ACh to α7 nAChR located on the terminal Schwann cells.[16]

Interestingly, the sole inherited human disease resulting from one nucleotide deletion (c.1497delG [p.Lys499Asnfs*13]) in *SLC5A7* already reported in the literature is DHMN7A with no signs of myasthenia.[6,23,24] The individuals reported here did not present any features of neuropathy at last examination, but we cannot exclude the development of neuropathic signs with age. The residual CHT activity in DHMN7A was estimated to be 25% in vitro, which is higher than that observed for the CMS-EA-associated mutations.[6] This may point to a relationship between the residual CHT activity and the associated phenotype that deserves further investigations.

CMS-EA is one of the most lethal CMS types due to the sudden episodes of apnea in neonates. The most frequent cause of obstructive apneas in newborns is a passive pharyngeal collapse during inspiration due to low muscle tone, which is probably favored by the congenital muscle weakness. Sudden apneic episodes have been sometimes reported in fast-channel syndrome and forms of CMS resulting from mutations in the genes encoding the skeletal muscle sodium channel and rapsyn.[25–28] However, sudden EA as a constant feature is specific to forms of CMS resulting from ChAT and CHT deficiency, highlighting a relationship between congenital dysfunction of choline homeostasis and EA. ACh deficit also has probable dysautonomic effects in line with the established importance of cholinergic pathways in the autonomic nervous system. This is supported by the tachycardia and hypertension in mice heterozygous for a null allele of *Slc5a7*.[29,30] In vivo administration of HC-3—the specific inhibitor of CHT—leads to respiratory paralysis from central dysfunction, and cholinergic agonists as well as AChE inhibitors exert significant effects on respiratory rhythm.[31,32] The strong link observed between CHT deficiency and the occurrence of sudden EA therefore underlines the still not well-characterized role of central cholinergic transmission in the fine control of respiratory function.[33]

CHT is present in central cholinergic neurons and cholinergic neurotransmission is important for cognitive and behavioral functions.[19] One hypomorphic polymorphism in *SLC5A7* (rs1013940 corresponding to the c.265A>G [p.Ile89Val] variation) is associated with pediatric attention deficit-hyperactivity disorder (ADHD [MIM: 143465]).[34] Among the individuals with CMS-EA due to recessive *SLC5A7* mutations reported here, three (5, 6, and 7) present cognitive deficits. If the multifactorial etiology of cognition must be considered before evoking any strong risk of cognitive or behavioral deficits in subjects with impaired CHT, our observation leads to the recommendation of carefully following up the subjects to detect and adequately manage any central phenotype due to CHT deficiency.

To summarize, our present work demonstrates the existence of a clinical spectrum resulting from CHT dysfunction that highlights the complexity of synaptic cholinergic metabolism and its genetics in human diseases with possible multisystem involvement that deserves further investigation.

Supplemental Data

Supplemental Data include a supplemental note, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2016.06.033.

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Web Resources

1000 Genomes, http://www.1000genomes.org
dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/
ExAC Browser, http://exac.broadinstitute.org/
GenBank, http://www.ncbi.nlm.nih.gov/genbank/
Human Splicing Finder, http://www.umd.be/HSF3/HSF.html
MutationTaster, http://www.mutationtaster.org/
OMIM, http://www.omim.org/
PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/
SIFT, http://sift.bii.a-star.edu.sg/

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