

Pathogenic mechanisms of *RAPSN* mutations in congenital myasthenic syndromes



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A thesis submitted for the degree of
Doctor of Philosophy
[Trinity term 2015]

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Abstract

Rapsyn is essential for clustering acetylcholine receptors (AChR) at the neuromuscular junction (NMJ). Congenital myasthenic syndrome (CMS) due to *RAPSN* mutations compromises neuromuscular transmission through a deficiency of AChR at the NMJ. *RAPSN*-CMS is autosomal recessive, and most patients harbour a common mutation p.Asn88Lys. A definitive genetic diagnosis for patients who do not carry the p.Asn88Lys allele can be challenging. In this thesis 10 novel variants in *RAPSN* are shown to impair AChR clustering *in vitro*, and are thus defined as pathogenic.

The properties of *RAPSN* mutations p.Val45M, p.Asn88Lys, p.Arg91Leu and p.Ala153Thr were studied and were found to be diverse, though common mechanisms were found to underlie the AChR deficiency. The mutations reduce the stability of rapsyn and the stability of AChR-rapsyn clusters formed on cultured myotubes. A unique missense mutation in the AChR δ -subunit encoding gene, p.Glu381Lys, phenotypically mimics *RAPSN*-CMS, and investigations revealed that this mutation also causes instability of the AChR clusters.

Salbutamol, a medication that is believed to stabilise NMJ, has recently been found to benefit AChR deficiency patients when used in combination with pyridostigmine. However, the mode of action of salbutamol at the NMJ is undefined. A pilot study was performed to evaluate the efficacy and mechanism of salbutamol therapy in a mouse model of AChR deficiency. Methodologies were established for the analysis of the effects of salbutamol and will provide the basis for a more detailed study of its beneficial action in disorders of neuromuscular transmission.

Acknowledgements

I would like to thank my supervisors, Professor David Beeson and Dr. Richard Webster for their constant availability, patience and support. Their mentoring and guidance have been crucial in my research and the completion of this thesis.

To Dr. Owain Howell and Dr. Margareta Nikolic who supervised my previous research projects and inspired me to undertake of this DPhil experience.

To Professor Christian Eggeling, Dr. Christoffer Lagerholm and Dr. Dominic Waithe for their generous advice on microscopy and image analysis.

To my family and friends, in particular, my parents and sister who have provided unreserved encouragement and support. Also Mary So for her guidance on producing the graphic illustrations presented in this thesis.

Finally to all members of the Neurosciences Group who throughout the years have made my time in Oxford enjoyable. In particular, I would like to thank Dr. Wei Liu and Miss Susan Maxwell for their help with molecular cloning, and Professor Angela Vincent and Professor Bethan Lang for their constructive criticism and suggestions that have helped me to become a better scientist.

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Abbreviations

The current nomenclature for clinical genetics describes amino acid changes according to their 3 letter codes, e.g. p.Asn88Lys. For simplicity, the previous nomenclature, e.g. N88K was used in this thesis.

Amino acid letter codes					
A	Ala	Alanine	M	Met	Methionine
C	Cys	Cysteine	N	Asn	Asparagine
D	Asp	Aspartic acid	P	Pro	Proline
E	Glu	Glutamic acid	Q	Gln	Glutamine
F	Phe	Phenylalanine	R	Arg	Arginine
G	Gly	Glycine	S	Ser	Serine
H	His	Histidine	T	Thr	Threonine
I	Ile	Isoleucine	V	Val	Valine
K	Lys	Lysine	W	Trp	Tryptophan
L	Leu	Leucine	Y	Tyr	Tyrosine

3, 4-DAP	3, 4-diaminopyridine
ACF7	acetylcholine
ACh	acetylcholinesterase
AChE	acetylcholine receptor
AChR	bovine serum albumin
ANOVA	analysis of variance
BSA	bovine serum albumin
BuTx	bungarotoxin
C-	carboxyl-
C-agrin	carboxyl-terminal rat agrin
cAMP	cyclic adenosine monophosphate
Cdk5	cyclin-dependent kinase 5
cDNA	coding DNA
CHX	cycloheximide
CMS	congenital myasthenic syndromes
ColQ	collagen Q
CRISPR	clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
DGC	dystrophin-glycoprotein complex
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
Dok-7	docking protein-7
DSB	double-stranded DNA breaks
EDL	extensor digitorum longus muscle
EGFP	enhanced green fluorescent protein
EMG	electromyography
EPP	endplate potential
ER	endoplasmic reticulum
F-actin	filamentous actin
FCS	fetal calf serum
FL-agrin	full-length human agrin
HDR	homology-directed repair
HEK 293	human embryonic kidney cells
HRP	horseradish peroxidase
HS	horse serum
HSP90 β	heat shock protein 90 β
IRES	internal ribosome entry site
KO	knockout

LB	lysogeny broth
LG	laminin-globular domains
LRP-4	low density lipoprotein receptor-related protein 4
MuSK	muscle specific kinase
N-	amino-
Na _v 1.4	voltage-gated sodium channel
NGS	next generation sequencing
NHEJ	non-homologous end joining
NMJ	neuromuscular junction
PAM	protospacer adjacent motif
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEI	polyethylenimine
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SFK	Src family kinase
SNAP25	synaptosomal-associated protein
TA	tibialis anterior
TMR	tetramethylrhodamine
TPRs	tetratricopeptide repeats
trancRNA	transactivating CRISPR RNA
WT	wild type
sgRNA	single-guide RNA

Chapter 1

Introduction

1.1 Basic principles of neuromuscular transmission

The neuromuscular junction (NMJ) is a synapse formed between the motor nerve terminal and the postsynaptic membrane of the skeletal muscles (Figure 1-1). Neuromuscular transmission relies on the release of the neurotransmitter acetylcholine (ACh) from the nerve terminal and its interaction with acetylcholine receptors (AChR), a ligand-gated ion channel on the muscle membranes, to induce muscle contractions (Ruff, 2011; Stroud et al., 1990). Upon the arrival of the action potential, voltage-gated calcium channels at the nerve terminal open briefly to allow influx of calcium ions (Ca^{2+}), which result in the release of ACh from synaptic vesicles into the synaptic cleft. The interaction between ACh and AChR on the postsynaptic membrane leads to the opening of the receptor channel pores so that influx of cations into the muscle generates a localised depolarisation that is known as the endplate potential (EPP). Once the depolarisation of the postsynaptic membrane reaches the critical threshold, the voltage-gated sodium channels ($\text{Nav}1.4$) open and generate an action potential that propagates to cause muscle contractions (Figure 1-2).

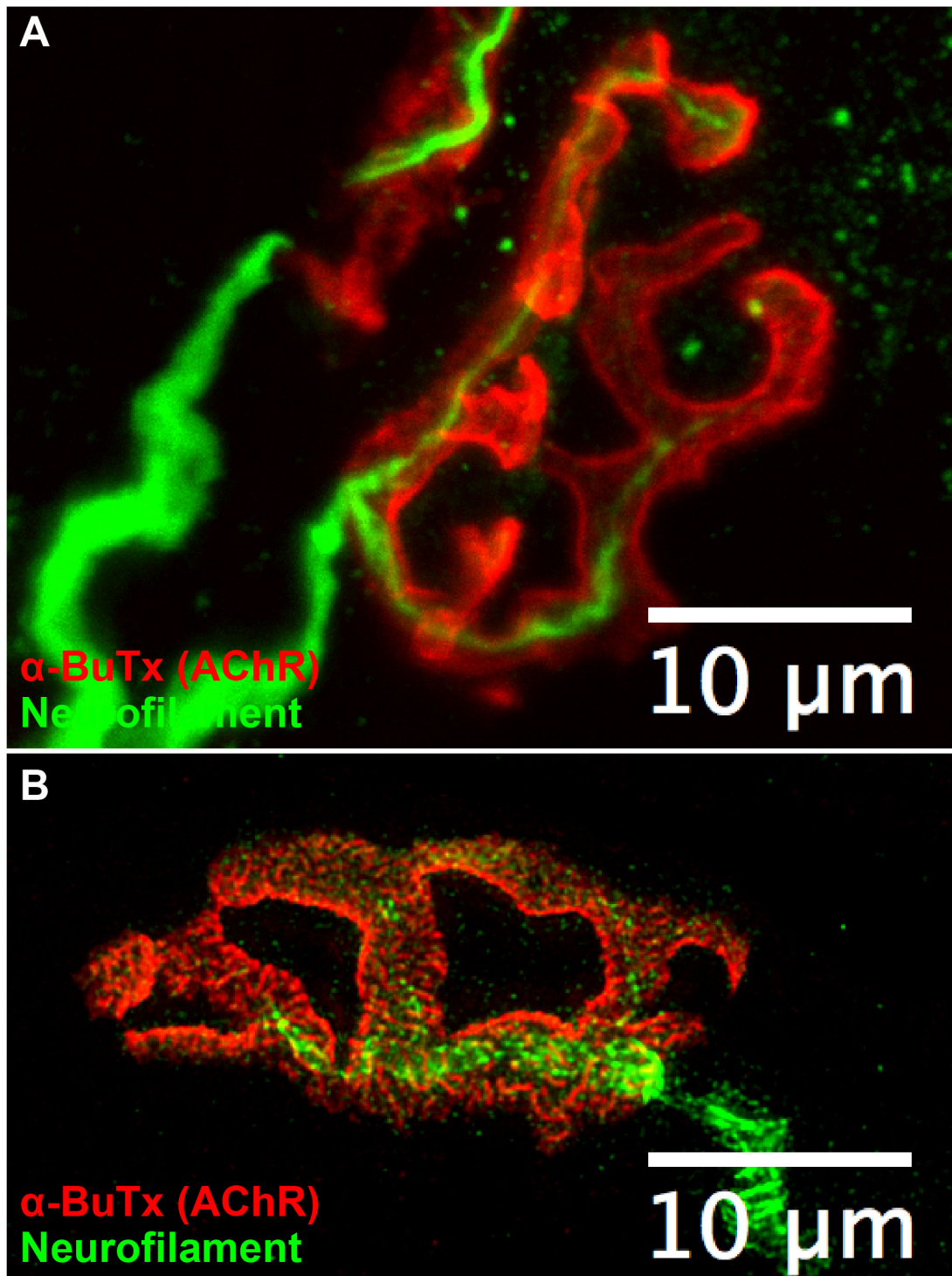


Figure 1-1 Maximum intensity projection images of the neuromuscular junction

The images show the neuromuscular junction (NMJ) in an adult mouse. AChR and the axons of motor neurons at the NMJs are labeled with α -bungarotoxin-TMR (α -BuTx-TMR, red) and an anti-neurofilament antibody (green). Images were acquired by (A) confocal microscopy and (B) stimulated emission depletion microscopy, which shows the concentration of AChR on the crests of the synaptic folds

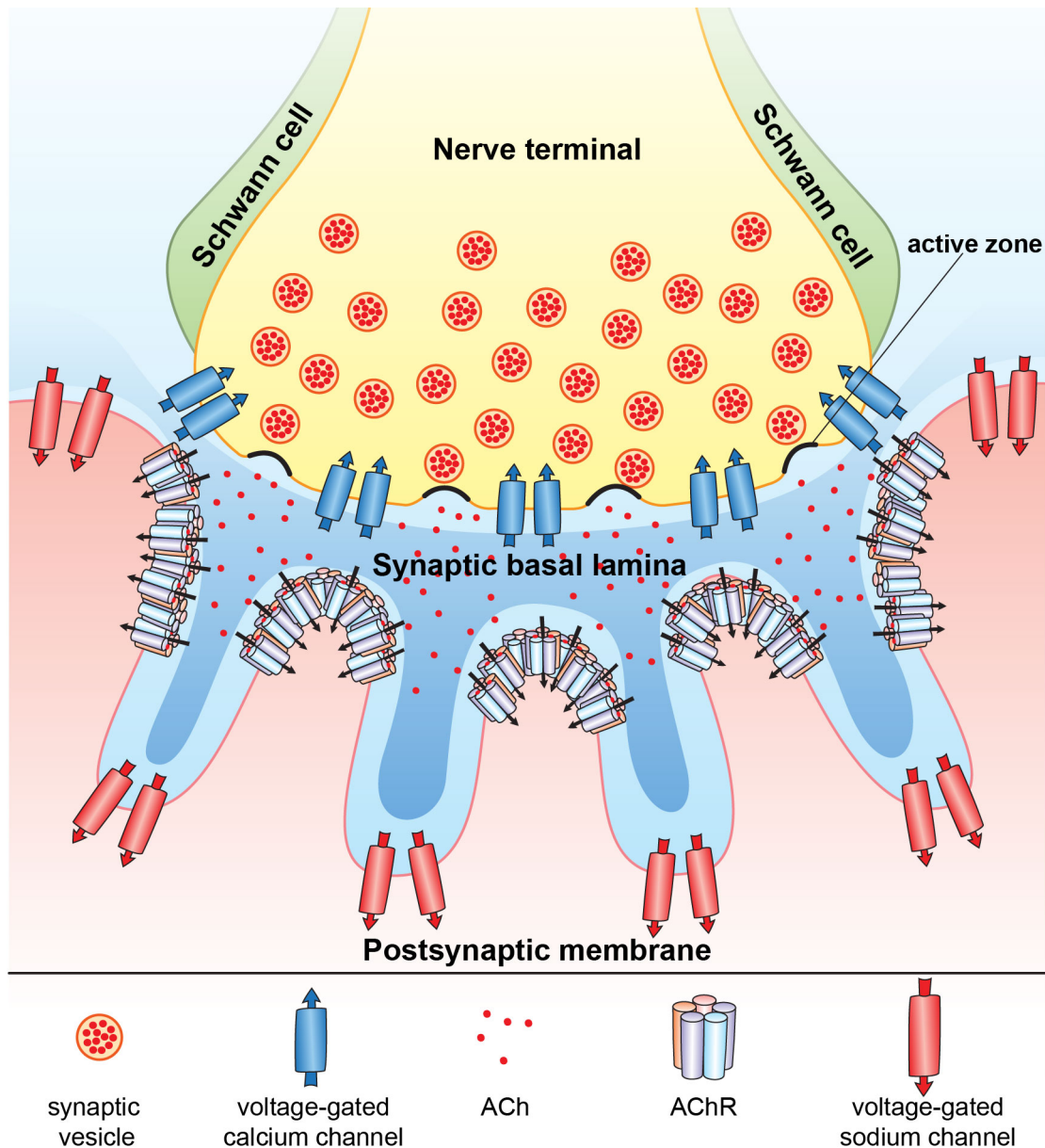


Figure 1-2 Schematic representation of neuromuscular transmission

At the presynaptic nerve terminal, the arrival of an action potential leads to the opening of the voltage-gated calcium ion channels, and the influx of Ca^{2+} ions leads to the release of acetylcholine (ACh) from the synaptic vesicles at the active zones. ACh binds to the acetylcholine receptors (AChR) clustered on the crests of the postsynaptic folds and opens the cationic channel pores on the receptors, which leads to localised depolarisation of the muscle membrane. Subsequently, if the required threshold is reached, the voltage-gated sodium channels are opened to allow for the influx of Na^{+} ions to generate an action potential that propagates along the muscle fibre, which ultimately leads to muscle contraction.

*Acetylcholinesterase (not shown) in the synaptic basal lamina rapidly hydrolyses ACh to terminate signal transmission.

1.2 Factors influencing neuromuscular transmission

Neuromuscular transmission utilises highly specialised architecture and mechanisms to trigger EPP that exceeds the required threshold for the generation and propagation of action potentials that cause muscle contraction (Fagerlund and Eriksson, 2009). Gene mutations in congenital myasthenic syndromes (CMS) compromise the safety factor of neuromuscular transmission, which is defined by the ratio between the amplitudes of actual EPP and EPP required for action potentials (Engel, 2007; Ruff and Lennon, 2008; Wood and R. Slater, 2001). This section provides an overview of the structural and functional features of the NMJ that influence the safety factor.

1.2.1 Presynaptic specialisation of the NMJ

The nerve terminal is the demyelinated part of the motor neuron that is branched and encapsulated by the terminal Schwann cell (Fagerlund and Eriksson, 2009). The synthesis of ACh from acetyl coenzyme A and choline is catalysed by choline acetyltransferase (*CHAT*) (Govindasamy et al., 2004), and ACh are packaged in vesicles by the vesicular acetylcholine transporter (Song et al., 1997). The vesicles mainly accumulate in proximity to the active zone, the site of exocytosis, of the presynaptic membrane (Nishimune, 2012; Rizzoli and Betz, 2004). The Ca^{2+} -triggered exocytosis of ACh requires cooperative actions between the synaptic vesicle-associated calcium sensor, synaptotagmin (*SYT*) expressed on vesicles (Fagerlund and Eriksson, 2009; Martyn et al., 2009) and the complex of 3 proteins within the group of soluble N-ethylmaleimide-sensitive-factor attachment receptor (SNARE) proteins: synaptobrevin on synaptic vesicles, and syntaxin and SNAP25 (*SNAP25B*) on the plasma membrane (Lang and Jahn, 2008).

1.2.2 Synaptic specialisation of the NMJ

In the synaptic cleft, the accumulation of ACh is regulated by acetylcholinesterase (*AChE*) (Shi et al., 2012), as prolonged exposure to ACh would desensitise AChR and prevent the opening of channel pores upon further ACh binding (Ruff, 1986). Approximately ~50% of the ACh quanta released and ACh dissociated from AChR are hydrolysed into acetate and choline rapidly by the acetylcholinesterase to terminate their actions (Martyn et al., 2009). Collagen Q (*COLQ*, ColQ) anchors and accumulates acetylcholinesterase at the synaptic basal lamina by its interactions with perlecan, a heparin sulfate proteoglycan that binds to laminin in the synaptic basal lamina, and the muscle specific kinase (MuSK), which is on the postsynaptic membrane (Cartaud et al., 2004; Kimbell et al., 2004).

Laminin and collagen are ubiquitous components of the basal lamina that are expressed at the neuromuscular synapse in specific isoforms: laminin- 221 ($\alpha 2\beta 2\gamma 1$), 421 ($\alpha 4\beta 2\gamma 1$) and 521 ($\alpha 5\beta 2\gamma 1$), and collagen IV ($\alpha 3$ - $\alpha 6$) chains, XIII and collagen Q (Shi et al., 2012). The synaptic basal lamina plays important roles in the development and maintenance of the structural integrity of the NMJ. Laminin $\beta 2$ (*Lamb2*) knockout mice possess aberrant presynaptic structures with reduced active zones and simplified postsynaptic folds (Noakes et al., 1995). Furthermore, the laminin-421 and collagen XIII are critical for the precise alignment of the presynaptic active zones and opening of the postsynaptic folds (Latvanlehto et al., 2010; Patton et al., 2001); whereas collagen IV ($\alpha 3$ - $\alpha 6$) is required for the maintenance of mature NMJ (Fox et al., 2007). On the other hand, in addition to its interaction with acetylcholinesterase, ColQ has been suggested to be involved in the processes for postsynaptic specialisation (Karmouch et al., 2013).

1.2.3 Postsynaptic specialisation of the NMJ

Across the synaptic cleft, the postsynaptic membrane is heavily invaginated and forms synaptic folds (Sanes and Lichtman, 2001). The openings of the postsynaptic folds are located immediately opposite to the presynaptic active zones, and AChR are tightly clustered on the crests of these folds (Fertuck and Salpeter, 1974; Slater, 2008). The highly ordered localisations of the presynaptic active zones and AChR at the NMJ are structural determinants for efficient neuromuscular transmission (Slater, 2008). The Na_v1.4 are located at the depths of the synaptic folds and also expressed along the muscle membrane, with higher density in proximity to the NMJ to amplify the responses to depolarisation and to enhance signal transductions that leads to muscle contraction (Cohen-Cory, 2002; Fagerlund and Eriksson, 2009).

1.2.3.1 Acetylcholine receptors (AChR)

There are 2 forms of muscle AChR that are expressed on the postsynaptic membrane: the foetal form of AChR comprises the α - (*CHRNA1*), β - (*CHRNBI*), δ - (*CHRND*) and γ - (*CHRNA1*) subunits, where the γ -subunit is replaced by the ϵ -subunit (*CHRNE*) in the adult form of the receptors (Mishina et al., 1986). Assembled AChR are pentamers that follow the composition of 2 α : 1 β : 1 δ : 1 γ / 1 ϵ (Unwin, 2005).

Each of the subunits has 3 key distinguishable regions: 1) a large N-terminal extracellular domain that consists of mostly β -strands and an α -helix; 2) a channel pore forming domain composed of 4 transmembrane α -helices (M1-M4) connected by the extracellular M2/M3 linker and intracellular M1/M2 and M3/M4 linkers; and 3) a short extracellular C-terminus domain (Unwin, 2005) (Figure 1-3).

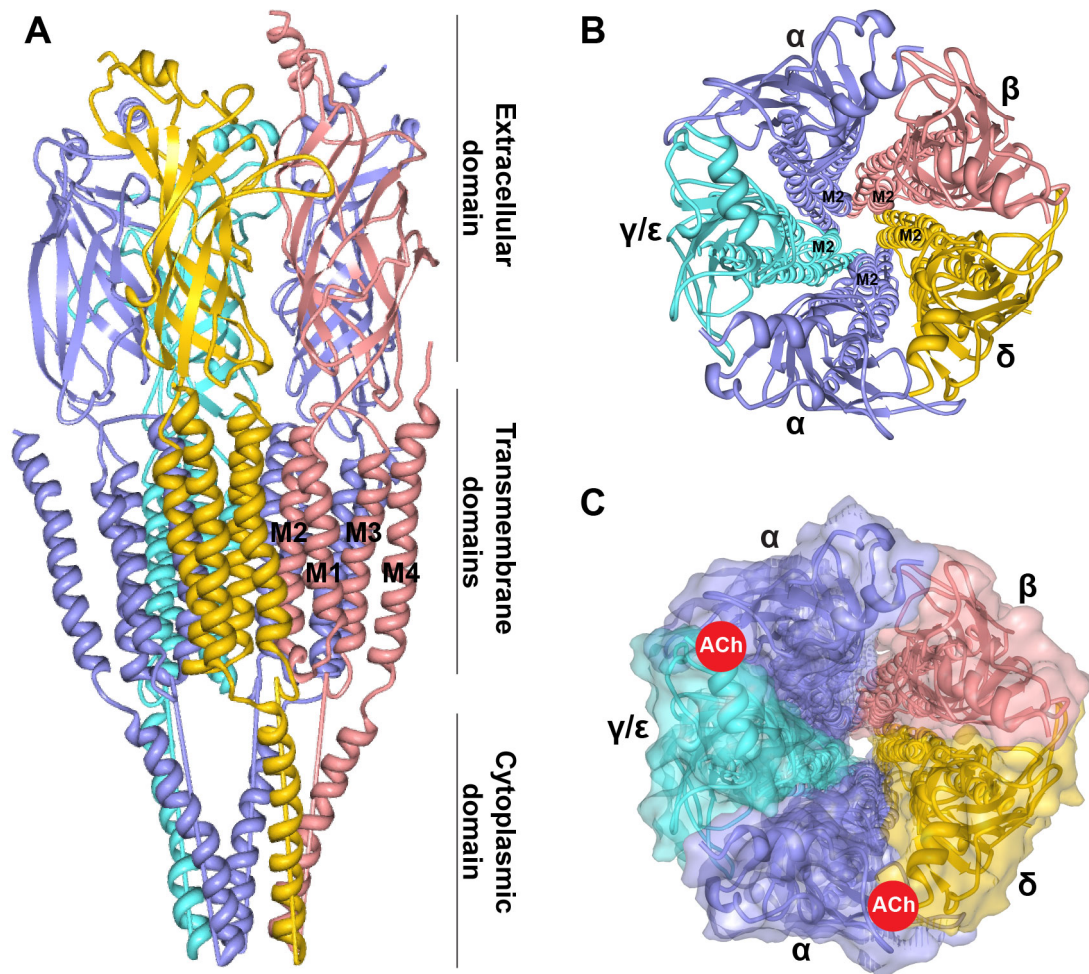


Figure 1-3 Three-dimensional structure of the AChR

(A) Ribbon diagram of AChR (side-view). Each of the 5 subunits has an extracellular domain composed mostly of β -strands and an α -helix, 4 transmembrane domains composed of 4 α -helices (M1-M4) on each of the subunits, and a large M3/M4 linker intracellular region, also known as the large cytoplasmic loop.

(B) Ribbon diagram of AChR (top-view). The M2 transmembrane domains of each of the subunits align to form the cation-permeable channel pore of the AChR.

(C) There are 2 ACh-binding sites on the AChR, which are located at the interfaces between the α -subunits and the δ - and γ/ϵ -subunits at their extracellular domains.

The images were adapted and modified from the protein data bank (PDB ID: 2BG9), (Unwin, 2005)

To execute its function as a ligand-gated ion channel, the M2 α -helices from each of the 5 subunits align to form the central cationic pore (Unwin, 2005). Binding of 2 ACh molecules to an AChR opens the channel pore to allow the influx of cations which leads to depolarisation of the postsynaptic membrane (Lingle et al., 1992). The interfaces between the extracellular domains of the α -subunits and their adjacent δ - or γ -/ ϵ - subunit form the 2 binding sites for ACh (Unwin, 2005). In addition, the M3/M4 linkers of the subunits, also known as the large cytoplasmic domains, interact with intracellular components and are important for the trafficking and localisation of AChR on the membrane surfaces (Lee et al., 2009; Rudell et al., 2014) (Figure 1-3).

Numerous regulatory checkpoints in the assembly and trafficking of AChR ensure that only correctly assembled pentamers are expressed on the cell surface. Studies on mouse muscle cells showed that only ~30% of the synthesised α -subunits are incorporated into pentamers (Merlie and Lindstrom, 1983). The assembly of AChR occurs in the endoplasmic reticulum (ER), and while assembled AChR pentamers are exported for further modifications, partially assembled intermediates and misfolded subunits are retained and degraded by ER-associated degradation (Christianson and Green, 2004). This discrimination process is mediated by an ER retention signal on the M1 helical domains of each of the AChR subunits that are masked in assembled pentamers (Wang et al., 2002). In addition, another retention motif on the cytoplasmic loop of the α -subunit is also thought to be involved (Keller et al., 2001). After export from the ER to Golgi apparatus, as an additional regulatory step, Golgi retention motifs present on the large cytoplasmic loops of the β - and δ - subunits have been identified to regulate the trafficking of AChR to the surface of myotube membranes (Rudell et al., 2014).

1.2.3.2 Agrin/MuSK/Lrp4/Dok-7 induced AChR clustering

The concentration of AChR in clusters at the crests of the postsynaptic folds is crucial for efficient neuromuscular transmission. On adult postsynaptic membrane, the density of AChR is over 1000-fold higher than the extra synaptic sites (Fambrough, 1979). In the early stages of synaptogenesis, spontaneous aneural AChR clusters are formed through pre-patterning, a process that is thought to be involved in defining sites for synapse formation (Panzer et al., 2006). However, it is noteworthy that synaptic contacts between the nerve and muscle can also be formed at regions where the pre-patterned clusters are absent (Lin et al., 2008). The subsequent formation and maturation of AChR clusters is dependent on signaling events governed by the muscle specific kinase MuSK (Wu et al., 2010). Although the signalling pathways involved in the clustering process are not clear, key players have been identified to act in the agrin-induced AChR clustering mechanism to cluster and maintain AChR clusters on the postsynaptic membrane.

Agrin (*AGRN*), derived from the nerve terminal, is a component of the synaptic basal lamina that is important for the postsynaptic differentiation and stabilisation through the activation of MuSK (Bezakova and Ruegg, 2003). AChR clustering in agrin deficient mice is markedly reduced, and these mice die before birth due to disrupted neuromuscular function (Gautam et al., 1996). Agrin is a large heparin sulfate proteoglycan (Tsen et al., 1995), and its C-terminal region, which contains 3 laminin-globular (LG1-3) domains, is crucial and sufficient for AChR clustering (Bezakova and Ruegg, 2003). It has been shown that, albeit at a lower potency, the LG3 domain alone can activate MuSK and induce AChR clustering (Gesemann et al., 1995).

The activation of MuSK begins with the binding of agrin to the low-density lipoprotein related protein 4 (LRP4; *LRP4*), and subsequently a 2:2 hetero-tetramer of agrin and LRP is formed (Kim et al., 2008; Zong et al., 2012). The interaction between the agrin-LRP4 complex and MuSK promotes the dimerisation and *trans*-autophosphorylation on the Tyr553 residue of MuSK (Watty et al., 2000; Zhang et al., 2011; Zong et al., 2012). The phosphorylated MuSK then recruits its muscle-intrinsic activator, docking protein-7 (Dok-7; *DOK7*), which is a non-catalytic adaptor protein (Bergamin et al., 2010; Okada et al., 2006). The binding of Dok-7 dimer, via its phosphotyrosine-binding domain, to phosphorylated MuSK further phosphorylates MuSK and activates MuSK kinase activities that orchestrates the clustering and maintenance of AChR clusters (Bergamin et al., 2010; Inoue et al., 2009).

The AChR clustering process in mice that are deficient in *Lrp4*, *Musk* or *Dok7* are more severely impaired than *Agrn* deficient mice as they lack AChR clusters completely and do not survive (DeChiara et al., 1996; Okada et al., 2006; Weatherbee et al., 2006). Therefore LRP4, MuSK and Dok-7 are essential for the formation of AChR clusters. Dok-7 is able to activate MuSK in the absence of agrin (Inoue et al., 2009; Kim et al., 2008; Zhang et al., 2011). In cultured myotubes, the overexpression of Dok-7, without agrin stimulation, induces phosphorylation of MuSK and leads to the formation of AChR clusters (Okada et al., 2006). Therefore LRP4/MuSK/Dok-7-induced signalling could also be responsible for the formation of aneural spontaneous AChR clusters formed in pre-patterning in the early stages of development (Bergamin et al., 2010). The pathways downstream of Dok-7 are unclear, but regardless of this, it is well known that rapsyn is another essential player that directly interacts with AChR to form clusters (Gautam et al., 1995).

1.3 Rapsyn

To understand the possible mechanisms of *RAPSN* mutations in congenital myasthenic syndromes, existing knowledge on rapsyn is described in greater detail.

Rapsyn (*RAPSN*), also known as the 43kDa receptor-associated protein of the synapse, is a postsynaptic membrane protein that colocalises with AChR at the neuromuscular junction (NMJ) (Figure 1-4). It is crucial for the formation and maintenance of AChR clusters on the postsynaptic membrane (Froehner et al., 1990; Witzemann, 2006). AChR clusters failed to form in *Rapsn* knockout mice and these mice die at birth without functional NMJ synapses (Gautam et al., 1995). Under the influences of agrin/LRP4/MuSK/Dok7 signalling, rapsyn clusters AChR (Froehner et al., 1990; Fuhrer et al., 1999), and acts as a linker between AChR and the postsynaptic cytoskeleton (Cartaud et al., 1998).

Cryoelectron tomography studies showed that within AChR clusters, up to 3 rapsyn molecules can complex with the cytoplasmic domain of a single receptor (Zuber and Unwin, 2013). The 3-dimensional crystallographic structure of rapsyn is not currently available. The cDNA clone of human rapsyn was sequenced nearly 2 decades ago (Buckel et al., 1996), and its amino acid (aa) sequence and functional experiments that involved recombinant rapsyn fragments have led to the proposal of distinctive functional domains in rapsyn (Bartoli et al., 2001; Frail et al., 1988; Ponting and Phillips, 1996; Ramarao and Cohen, 1998; Ramarao et al., 2001).

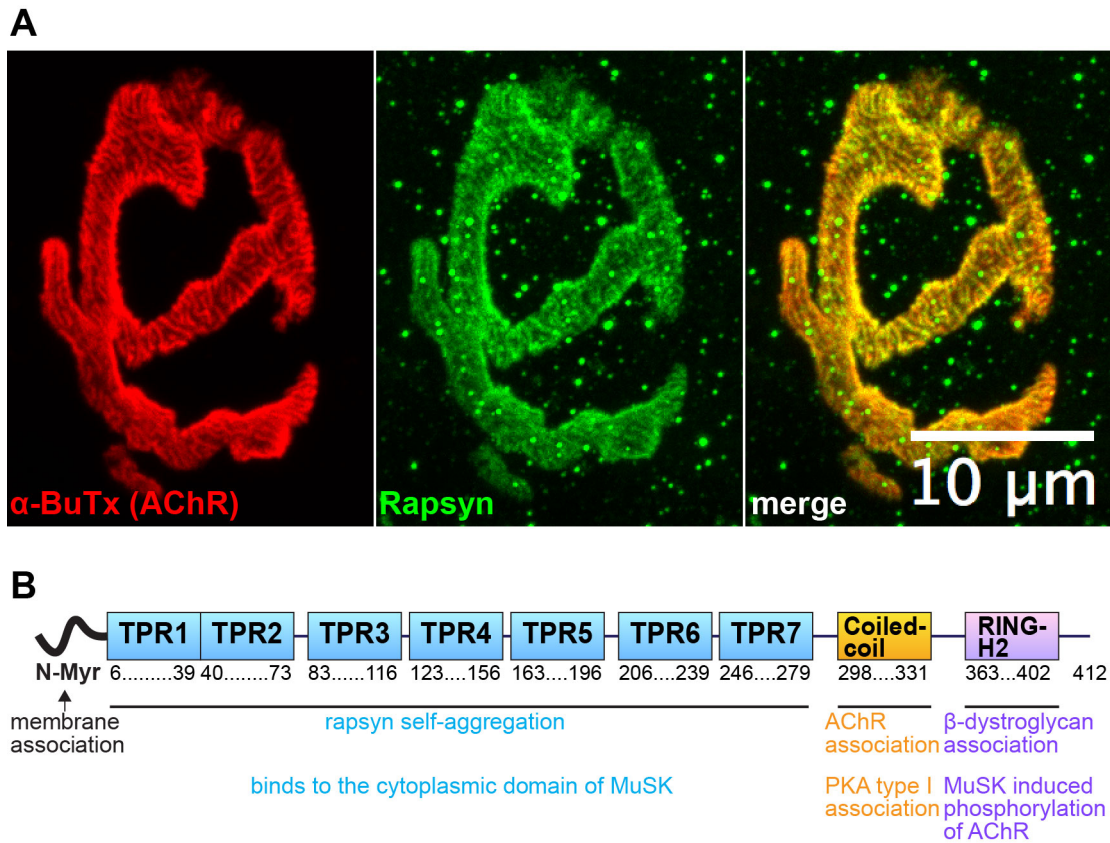


Figure 1-4 Localisation of AChR and rapsyn on the postsynaptic membrane

A) Neuromuscular junction (NMJ) in an adult mouse. AChR and rapsyn at the NMJs are labelled with α -bungarotoxin (α -BuTx-TMR, red) and an anti-rapsyn antibody, which was labelled with the secondary AlexaFluor-488 antibody (green). Images were acquired by confocal microscopy and are presented as maximum intensity projection images. * Signals in the green channel that are absent in the red channel are non-specific binding of antibodies to the sample preparation.

B) Rapsyn is predicted to possess 4 distinct functional domains: a N-myristylation (N-Myr) signal, 7 tetratricopeptide (TPR) repeats, a coiled-coil domain and a RING-H2 domain. The associated functions of the domains are also highlighted.

The amino (N)-terminus of rapsyn is myristoylated, a signal that is required for targeting rapsyn to the postsynaptic membrane (Frail et al., 1988). Rapsyn (aa6-361) is predicted to form eight tetratricopeptide repeats (TPRs) (Ponting and Phillips, 1996). It has been suggested that only 2 TPRs would suffice for rapsyn self-association (Ramarao and Cohen, 1998). In literature, rapsyn is often said to have seven TPRs (aa6-279) instead because a putative zinc finger/coiled-coil domain (aa298-331) that overlaps with the 8th TPR was demonstrated to be required for rapsyn-AChR association (Ramarao and Cohen, 1998; Ramarao et al., 2001). The RING-H2 domain (aa363-402) anchors rapsyn towards cytoskeleton proteins such as β -dystroglycan (Bartoli et al., 2001). Adjacent to the RING-H2 domain (aa403-406) of rapsyn, there is a consensus sequence for phosphorylation by PKA and PKC (Ramarao and Cohen, 1998). Further studies have identified that the RING-H2 domain of rapsyn is required for the MuSK-induced phosphorylation of AChR, a process that regulates AChR localisation (Antolik et al., 2006). However, the predicted functional domains on rapsyn are based on deletion studies that only highlight the regions that are crucial for their corresponding function, but the importance of residues at other sites within the protein cannot be excluded.

1.3.1 Interactions between rapsyn and AChR

When individually overexpressed in heterologous cells, all of the AChR subunits can interact with rapsyn, and the interaction requires the major cytoplasmic loop of the receptor subunits (Huebsch and Maimone, 2003; Maimone and Merlie, 1993; Maimone and Enigk, 1999). On the other hand, studies that used assembled chimeric subunits have suggested that the cytoplasmic loops of δ - and ϵ - subunits are not required for AChR clustering by rapsyn (Yu and Hall, 1994b). It was later identified

that rapsyn preferentially interacts with a putative α -helix that is present in the cytoplasmic loop of β -subunit, followed by ε and α , but not with the δ -subunit due to its shorter α -helix (Ito et al., 1997; Le Novere et al., 1999; Lee et al., 2009).

In the agrin induced MuSK signalling cascade, phosphorylation of AChR precedes AChR clustering (Wallace et al., 1991). The tyrosine phosphorylation of the Tyr390 residue at the large cytoplasmic loop of the β -subunit (β^{Tyr390}) of the AChR increases binding of rapsyn to the receptor (Borges et al., 2008); and the ablation of tyrosine phosphorylation of the β -subunit modestly impairs the clustering and anchoring of AChR in mice (Friese et al., 2007). Furthermore, the phosphorylation of β^{Tyr390} increases the half-life of AChR in cultured myotubes, which contributes to the stability of the AChR clusters (Rudell and Ferns, 2013).

Rapsyn facilitates the phosphorylation of β^{Tyr390} , which promotes the anchorage of AChR to the cytoskeleton (Lee et al., 2008). This mode of action can be achieved via Src-family kinases (SFK)-induced phosphorylation (Mohamed and Swope, 1999). However, after agrin stimulation, SFK only initially acts to phosphorylate MuSK and AChR, but its action is not required for the formation of AChR clusters as it is later compensated by Abl kinases (Mittaud et al., 2004; Smith et al., 2001). Instead, SFK activities stabilise the postsynaptic apparatus by maintaining the interactions between AChR, rapsyn and the cytoskeleton (Sadasivam et al., 2005). Therefore the involvement of rapsyn in mediating the activities of SFKs in turn strengthens its interaction with AChR as well as the metabolic stability of the receptors, and enhances the stability of the AChR clusters.

1.3.2 Interactions between rapsyn and the cytoskeleton

AChR clustering is also dependent on the cytoskeleton, as interference to the assembly of filamentous (F)-actin reduces the number of AChR clusters formed (Dai et al., 2000). Rapsyn has been found to interact with several scaffolding proteins of the cytoskeleton; however, the precise cytoskeletal network involved in the specialised architecture of the postsynaptic membrane has not been defined.

The spectrin superfamily is composed of proteins that scaffold F-actin, and each member possesses an actin-binding domain and multiple spectrin-like repeats (Nicolas et al., 2014). Within this family, α -actinin and ACF7 have been identified to interact with rapsyn directly (Antolik et al., 2007; Dobbins et al., 2008). Rapsyn is also thought to bind to β -dystroglycan (Bartoli et al., 2001), a protein that is known to interact with utrophin (another spectrin superfamily protein) (Chung and Campanelli, 1999). Furthermore, the interaction between β -catenin and rapsyn could provide additional anchorage of AChR clusters, via α -catenin, to α -actinin and F-actin (Nieset et al., 1997; Zhang et al., 2007). Hence the anchorage of AChR clusters to the actin cytoskeleton may be mediated by direct/indirect interactions between rapsyn and proteins of the spectrin superfamily and β -catenin.

In addition to actin, AChR clusters are also anchored to the desmin intermediate filament network in the cytoskeleton by the interaction between the plectin isoform 1f and rapsyn (Mihailovska et al., 2014). Compared to F-actin, desmin intermediate filaments are more flexible and tensile (Kreplak et al., 2008; Mucke et al., 2004). Hence the anchorage of AChR clusters to intermediate filaments may provide additional stability to AChR clusters under muscle contraction. Plectin 1f also

interacts with β -dystroglycan (Rezniczek et al., 2007), which highlights the likelihood of the interacting partners of rapsyn forming a scaffold together in the cytoskeleton.

Of note, the number of AChR clusters formed on myotubes deficient in dystroglycan or plectin is reduced (Jacobson et al., 2001; Mihailovska et al., 2014); and the suppression of the expression of α -actinin or β -catenin also had similar consequences (Dobbins et al., 2008; Zhang et al., 2007). Therefore the interactions between rapsyn and the components of the cytoskeleton are not crucial for the formation of AChR clusters, but are more involved in the localisation, maturation and maintenance of AChR clusters instead.

Figure 1-5 shows a schematic summary on molecular architecture of the postsynaptic membrane.

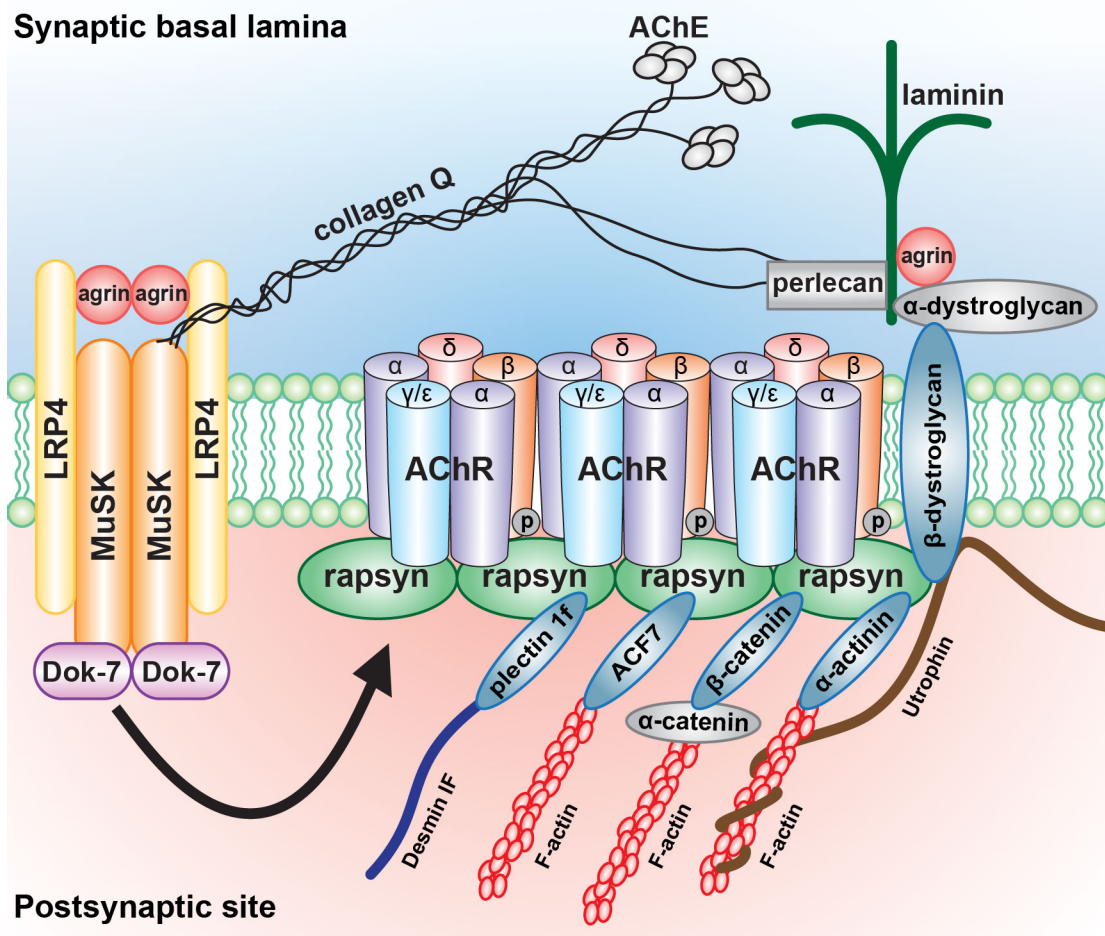


Figure 1-5 Schematic representation of the molecular architecture of AChR clusters on the postsynaptic membrane

The clustering of AChR on the postsynaptic membrane is orchestrated by the agrin/MuSK/LRP4/Dok-7 signal cascade.

Agrin released from the nerve terminal is localised to the synaptic basal lamina via its interaction with laminin. It binds to LRP4 and forms a 2:2 hetero-tetramer which promotes the dimerisation and autophosphorylation of MuSK. The interaction between Dok-7 and MuSK is also required for the activation of the downstream signalling events that cause rapsyn to cluster AChR.

Numerous scaffolding proteins, including ACF7, α-actinin, plectin 1f and β-catenin have been identified to anchor rapsyn-AChR clusters to the postsynaptic cytoskeleton, contributing towards the maintenance of AChR clusters. The dystrophin-glycoprotein complex (DGC) also maintains the stability of the AChR clusters. The α- and β-dystroglycan, as well as utrophin of the DGC are shown. In addition, the interaction between laminin and α-dystroglycan is involved in the formation of the synaptic basal lamina. Perlecan binds to laminin, and together with MuSK localises collagen Q-AChE in proximity to synaptic sites as part of the specialised architecture of the NMJ.

1.4 Maintenance of AChR clusters

As AChR clusters mature, they increase in size and density, and their morphology undergoes transformation from plaque-like structures to more complex and perforated pretzel-like structures (Marques et al., 2000; Sanes and Lichtman, 2001). Agrin induced MuSK signalling is required for the maintenance of the NMJ (Samuel et al., 2012; Tezuka et al., 2014), as postnatal inactivation of MuSK leads to the loss of AChR and disassembly of the synapse (Hesser et al., 2006; Kong et al., 2004). Rapsyn is indispensable in the maintenance of AChR clusters, since it has been shown that silencing rapsyn in adult rat muscles reduced rapsyn and AChR expression at similar levels, suggesting rapsyn determines AChR density at the postsynaptic membrane (Martinez-Martinez et al., 2009).

The dystrophin glycoprotein complex (DGC) connects the synaptic basal lamina to the postsynaptic components and is important for the stabilisation of AChR clusters at the NMJ (Jacobson et al., 2001; Martinez-Pena y Valenzuela et al., 2011). It is composed of dystroglycan, dystrophin/utrophin, syntrophin and dystrobrevin (Banks et al., 2003). β -dystroglycan binds to extracellular α -dystroglycan, which interacts with laminin in the synaptic basal lamina (Banks et al., 2003). Laminin is involved in the maturation of AChR clusters (Kummer et al., 2004; Mazhar and Herbst, 2012). In addition, agrin binds to laminin for its localisation at the NMJ (Mascarenhas et al., 2003), and the depletion of agrin at the NMJ of adult mice results in the complete loss of AChR clusters (Samuel et al., 2012). Since the concentration of DGC proteins at the NMJ is dependent on rapsyn expression (Gautam et al., 1995), rapsyn can be involved in stabilising AChR clusters via any associated functions of the DGC.

1.5 Congenital myasthenic syndromes (CMS)

CMS are a group of inherited disorders caused by gene mutations that result in aberrant neuromuscular transmission (Palace and Beeson, 2008). Fatigable weakness in the skeletal muscle is a characteristic feature that commonly presents during infancy, but the severity and distribution of the myasthenic weakness are variable due to the different underlying mechanisms of CMS subtypes (Rodriguez Cruz et al., 2014). The prevalence of genetically confirmed CMS cases in the United Kingdom is estimated at 9.2 cases per million children under 18 years of age (Parr et al., 2014).

A generic diagnosis of CMS can be made on the basis of clinical presentation, and if available, the family history of patients and electromyographic (EMG) studies (Engel et al., 2015). It is crucial to distinguish CMS from the autoimmune myasthenia gravis to avoid unnecessary immunosuppressive therapy for patients (Finlayson et al., 2013). Genetic analysis provides a definitive diagnosis of CMS, and this is usually performed in a targeted manner based on clinical, familial and EMG assessments (Engel et al., 2015). CMS-subtypes are defined by their causative gene mutations and molecular mechanisms, and are traditionally classified according to their sites of defects into presynaptic, synaptic basal lamina-associated or postsynaptic CMS (Engel, 2007). Recently, mutations in enzymes of glycosylation processes have also been identified to compromise neuromuscular transmission and cause CMS (Belaya et al., 2012; Belaya et al., 2015; Cossins et al., 2013; Guergueltcheva et al., 2011). Since glycosylation enzymes are ubiquitously expressed and could alter the functions of NMJ-associated glycoproteins, they form a new class of CMS (Engel et al., 2015). To date, mutations in over 20 different genes have been identified to cause CMS (Table 1-1).

Table 1-1 Classification of congenital myasthenic syndromes

CMS subtype		Gene	References
Presynaptic			
Choline acetyltransferase deficiency		<i>CHAT</i>	(Ohno et al., 2001b)
PREPL deletion syndrome (ubiquitous)		<i>PREPL</i>	(Regal et al., 2014)
SNAP25B deficiency		<i>SNAP25B</i>	(Shen et al., 2014)
Synaptotagmin 2 deficiency		<i>SYT2</i>	(Herrmann et al., 2014)
Synaptic			
Agrin deficiency		<i>AGRN</i>	(Huze et al., 2009)
Acetylcholinesterase deficiency		<i>COLQ</i>	(Ohno et al., 1998)
Laminin-β2 deficiency		<i>LAMB2</i>	(Maselli et al., 2009)
Postsynaptic			
Defects in AChR	Primary AChR deficiency	<i>CHRNA1, CHRNB1, CHRND, CHRNE</i>	(Engel et al., 2003)*
	Fast-channel syndrome	<i>CHRNA1, CHRND, CHRNE</i>	(Engel et al., 2003)*
	Slow-channel syndrome	<i>CHRNA1, CHRNB1, CHRND, CHRNE</i>	(Engel et al., 2003)*
	Channel-conductance syndrome	<i>CHRNE</i>	(Webster et al., 2012)
	Escobar syndrome	<i>CHRNA1</i>	(Morgan et al., 2006)
Dok-7 myasthenia		<i>DOK7</i>	(Beeson et al., 2006)
Rapsyn deficiency		<i>RAPSN</i>	(Ohno et al., 2002)
LRP4 myasthenia		<i>LRP4</i>	(Ohkawara et al., 2014)
MuSK deficiency		<i>MUSK</i>	(Chevessier et al., 2004)
Sodium channel myasthenia		<i>SCN4A</i>	(Tsujino et al., 2003)
Plectin deficiency		<i>PLEC1</i>	(Banwell et al., 1999)
Glycosylation defects			
ALG2		<i>ALG2</i>	(Cossins et al., 2013)
ALG14		<i>ALG14</i>	(Cossins et al., 2013)
GFPT1		<i>GFPT1</i>	(Senderek et al., 2011)
DPAGT1		<i>DPAGT1</i>	(Belaya et al., 2012)
GMPPB		<i>GMPPB</i>	(Belaya et al., 2015)

The functional importance of the gene products of confirmed CMS cases associated with the presynaptic, synaptic and postsynaptic forms of CMS have been briefly discussed in the previous chapter. To supplement:

The molecular consequence of PREPL deficiency is not known, but it has been speculated to defect the filling of ACh to synaptic vesicles, which may compromise neuromuscular transmission (Regal et al., 2014).

The pathogenic consequence of glycosylation defects at the NMJ is currently unclear, and thus will not be discussed in detail.

* Reviews the CMS subtypes caused by mutations in genes encoding for AChR subunits.

1.5.1 *RAPSN*-CMS

Mutations in *RAPSN* cause a major subtype of congenital myasthenic syndromes (CMS), conferring approximately 20% of all CMS cases in the British Isles (Finlayson et al., 2013). *RAPSN*-CMS is autosomal recessive, with the common mutation, p.Lys88Asn (N88K), that is harboured in at least one allele of over 90% of the patients in the UK (Finlayson et al., 2013). Electron microscopy on biopsy specimen of *RAPSN*-CMS patients showed reductions in the number and density of AChR at simplified post-junctional folds, causing AChR deficiency at motor endplates (Engel, 2012; Ohno et al., 2002). Electromyographic (EMG) studies on the affected muscles of patients often identify decremental responses after repetitive nerve stimulation, suggesting reduced strength in synaptic transmission (Burke et al., 2003; Milone et al., 2009; Ohno et al., 2002). The aberrant clustering of the AChR by mutant rapsyn is likely to compromise neuromuscular transmission and result in the fatiguable muscle weaknesses of CMS.

Over 40 mutations have been identified in *RAPSN*; this includes different types of mutations that are dispersed across the entire rapsyn gene (summarised in Figure 1-6 and Tables 1-2 to 1-5). In some reports, the pathogenicity of novel mutations was predicted (Dunne and Maselli, 2003; Milone et al., 2009); in other cases, functional analyses were performed to demonstrate pathogenic mechanisms. Mutations at the E-box element within the *RAPSN* promoter region impair the transcriptional activities of *RAPSN* and are predicted to reduce rapsyn expression (Ohno et al., 2003). Frameshift and truncating mutations can result in the loss of functional domains in rapsyn and disrupt rapsyn functions (Banwell et al., 2004); in addition, nonsense mutations may ablate mutant protein expression through the nonsense mediated

mRNA decay mechanism (Frischmeyer and Dietz, 1999; Richard et al., 2003). Missense mutations can reduce rapsyn expression, impair rapsyn self-association, prevent rapsyn-AChR clustering or affect the stability of AChR clusters (Cossins et al., 2006; Muller et al., 2006a; Ohno et al., 2002; Vogt et al., 2008). There are also splice site mutations that may truncate the rapsyn peptide and result in a loss of function (Milone et al., 2009; Muller et al., 2006a). The molecular consequences of *RAPSN* mutations ultimately compromise rapsyn-AChR aggregation. Without the precise crystallographic structure of rapsyn being available, the determination of the pathogenicity of mutations relies upon functional analyses of AChR clustering by rapsyn variants *in vitro*.

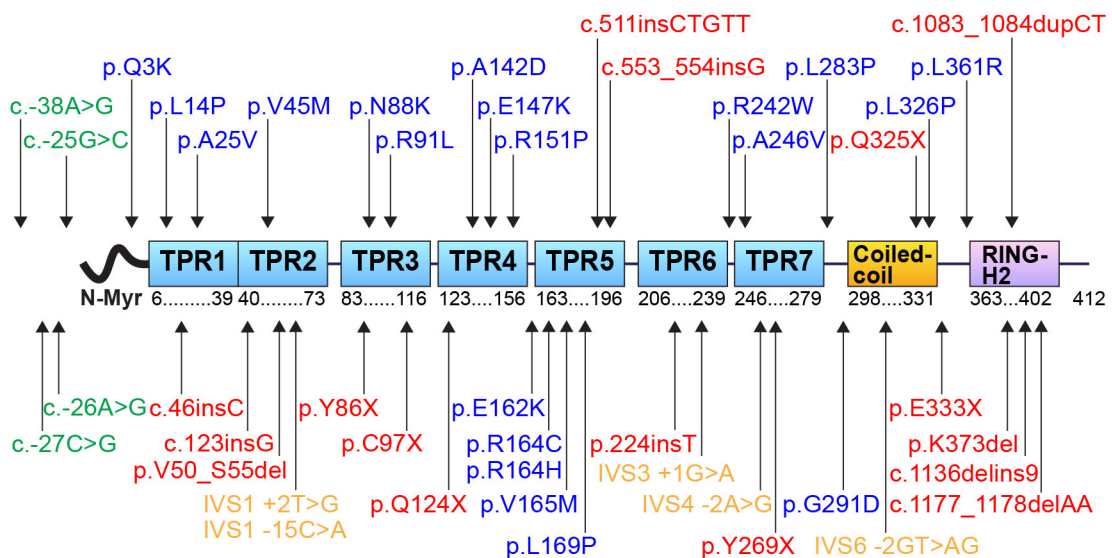


Figure 1-6 Summary of *RAPSN* mutations reported.

Green = promoter E-box mutations; Red = Insertion/deletion/nonsense mutations; Blue = Missense mutations; Orange = splice site mutations (mutations are intronic, and are illustrated based upon the locations of adjacent exons that corresponds to the domains) RefSeq accession: NM_005055/NP_005046

Table 1-2 Mutations in the E-box element of the promoter region in *RAPSN*

Promoter Ebox mutations			
Nucleotide change	Domain	Identified molecular consequences	References
-25G>C	Promoter	Reduce expression of rapsyn	(Ohno et al., 2003)*, (Ohno and Engel, 2004)
-26A>G	Promoter	Pathogenic on functional assay (unpublished observation)	(Beeson et al., 2008)*
-27C>G	Promoter	Pathogenic on functional assay (unpublished observation)	(Beeson et al., 2008)*
-38A>G	Promoter	Reduce expression of rapsyn	(Ohno et al., 2003)*, (Leshinsky-Silver et al., 2012)
The corresponding publications that show data from molecular analyses are marked with *			

Table 1-3 Splice site mutations in *RAPSN*

Splice site mutations				
Nucleotide change	Intron	Domain	Identified molecular consequences	References
IVS1+2T>G	1	TPR2	-	(Brugnoni et al., 2010)
IVS1-15C>A	1	TPR2	Aberrant RNA splicing, which causes frameshift and truncation	(Muller et al., 2006a)*
IVS3+1G>A	3	TPR6?	-	(Cossins et al., 2006)
IVS4-2A>G	4	TPR7	-	(Richard et al., 2003), (Ioos et al., 2004)
c.966+1GT>AG	6	Coiled-coil	-	(Milone et al., 2009)
The corresponding publications that show data from molecular analyses are marked with *				

Table 1-4 Insertion, deletion and nonsense mutations in *RAPSN*

Insertion, deletion & nonsense mutations					
Nucleotide change	Amino acid change	Exon	Domain	Identified molecular consequences	References
46insC	frameshift> truncation	1	TPR1	-	(Burke et al., 2003), (Dunne and Maselli, 2003)
123insG	-	1	TPR2	-	(Cossins et al., 2006)
-	V50_S55del	1	TPR2	-	(Milone et al., 2009)
-	Y86X (truncation)	2	TPR3	-	(Ioos et al., 2004)
-	C97X (truncation)	2	TPR3	-	(Ohno and Engel, 2004), (Cossins et al., 2006)
370C>T	Q124X (truncation)	2	TPR4	-	(Burke et al., 2003)
551insCTGTT	Frameshift> truncation	3	TPR5	-	(Burke et al., 2003)
553_554insGTTCT	Frameshift	3	TPR5	Impaired rapsyn-AChR clustering	(Ohno et al., 2002)*
673_676insACT	p.224insT	3	TPR6	-	(Leshinsky-Silver et al., 2012)
807C>A	Y269X (truncating)	5	TPR7	-	(Dunne and Maselli, 2003)
-	Q325X (truncating)	7	Coiled-coil	-	(Milone et al., 2009)
-	E333X (truncating)	7	Coiled-coil and RING-H2	-	(Banwell et al., 2004)
1083_1084dupCT	Frameshift> truncation	7	RING-H2	-	(Richard et al., 2003), (Ioos et al., 2004)
-	K373del*	7	RING-H2	Reduced rapsyn stability	(Cossins et al., 2006)*
1136delins9	-	7	RING-H2	-	(Cossins et al., 2006)
1177-1178delAA	Frameshift	8	RING-H2	Reduced rapsyn expression and impaired rapsyn-AChR clustering	(Burke et al., 2003), (Banwell et al., 2004), (Vogt et al., 2008)*
4.5kb chromosomal deletion		7-8	Coiled-coil and RING-H2	Loss of exon 7 and 8	(Muller et al., 2004)

The corresponding publications that show data from molecular analyses are marked with *

Table 1-5 Missense mutations in *RAPSN*

Missense mutations					
Nucleotide change	Amino acid change	Exon	Domain	Identified molecular consequences	References
7C>A	Q3K	1	PrecedeTPR1	-	(Muller et al., 2003)
41T>C	L14P	1	TPR1	Impaired rapsyn-AChR clustering	(Ohno et al., 2002)*, (Ohno and Engel, 2004), (Burke et al., 2003), (Dunne and Maselli, 2003)
-	A25V	1	TPR1	Reduced rapsyn stability; Impaired rapsyn-AChR clustering	(Cossins et al., 2006)*
133G>A	V45M	1	TPR1	Impaired rapsyn-AChR clustering	(Maselli et al., 2007)*
264C>A	N88K	2	TPR3	Impaired rapsyn-AChR clustering; Unstable AChR clusters	(Ohno et al., 2002)*, (Ohno and Engel, 2004), (Burke et al., 2003), (Maselli et al., 2003), (Richard et al., 2003), (Cossins et al., 2006)**, (Milone et al., 2009)
272G>T	R91L	2	TPR3	Impaired rapsyn-self association	(Burke et al., 2003), (Cossins et al., 2006)*
-	A142D	2	TPR4	-	(Ohno and Engel, 2004)
-	E147K	2	TPR4	-	(Milone et al., 2009)
-	R151P	2	TPR4	-	(Ohno and Engel, 2004)
284G>A	E162K	2	TPR4-TPR5	Impaired rapsyn-AChR clustering	(Maselli et al., 2007)*
-	R164C	2	TPR5	Impaired rapsyn-AChR clustering	(Muller et al., 2006a)*
-	R164H	2	TPR5	-	(Milone et al., 2009)
493G>A	V165M	2	TPR5	-	(Richard et al., 2003), (Cossins et al., 2006)
-	L169P	2	TPR5	-	(Cossins et al., 2006)
-	R242W	4	TPR6-TPR7	-	(Milone et al., 2009)
737C>T	A246V	4	TPR7	-	(Muller et al., 2003)
-	L283P	5	TPR7-coiled-coil	Impaired rapsyn-AChR clustering	(Muller et al., 2006a)*, (Cossins et al., 2006)
872G>A	G291D	5	TPR7-coiled-coil	-	(Muller et al., 2003)
-	L326P	7	Coiled-coil	-	(Milone et al., 2009)
1082_83TC>GT	L361R	7	Coiled-coil-RING-H2	Reduced rapsyn stability; Unstable rapsyn-AChR clusters	(Burke et al., 2003; Cossins et al., 2006)*

The corresponding publications that show data from molecular analyses are marked with *

The majority of *RAPSN*-CMS patients have an early onset and present symptoms at birth or during infancy (Burke et al., 2003; Milone et al., 2009); typical features include bulbar and respiratory weaknesses and muscle hypotonia (Finlayson et al., 2013). Multiple joint contractures and congenital malformation, both of which can be associated with foetal akinesia, affect nearly a third of the patients (Burke et al., 2003; Engel, 2012; Milone et al., 2009). Acute life-threatening episodic crises often occur in *RAPSN*-CMS patients during their infancy and early childhood (Finlayson et al., 2013). A few patients are affected by late-onset *RAPSN*-CMS, which resembles seronegative myasthenia gravis and symptoms do not present until adolescence or adulthood (Alseth et al., 2011; Burke et al., 2003; Cossins et al., 2006).

The early years may be a critical period for most patients as they often show clinical improvements with age and can eventually become minimally symptomatic (Burke et al., 2003; Maselli et al., 2003). The presence of both early and late onset cases in patients who harbour the common mutation, N88K, suggests a variation in the natural history of *RAPSN*-CMS (Burke et al., 2003; Cossins et al., 2006; Muller et al., 2003). Most patients with homozygous N88K mutation have a relatively mild disease course (Maselli et al., 2003; Muller et al., 2003), with some reported to be asymptomatic (Maselli et al., 2003). It is worth noting that a phenotypic spectrum, which ranges from respiratory failure at birth through to mild limb weaknesses throughout life, exists within N88K homozygosity (Milone et al., 2009). More severe phenotypes have been described in patients that are heteroallelic for N88K with truncating mutations (Cossins et al., 2006; Maselli et al., 2003; Richard et al., 2003), but one study failed to identify such an association (Milone et al., 2009). More cohort studies may better define the natural history of the disease.

Homozygous mutation, -38E>A, at the promoter E-box region of *RAPSN* (shown to arise from a common founder in Near Eastern population) was found to be associated with facial dysmorphism, marked by jaw deformities (Milone et al., 2009; Ohno et al., 2003). Truncating mutations in *RAPSN* have been suggested to be associated with increased risk of congenital arthrogryposis (Beeson et al., 2005), but this has not been consistently identified (Milone et al., 2009; Muller et al., 2006a). No other correlations between mutations, including N88K, and severity or phenotypic features have been found, highlighting the rare, yet heterogeneous, nature of the disease.

Episodic respiratory crises in association with or without infections can intermittently worsen the disease course of patients that originally present with varying clinical severity (Beeson et al., 2005; Milone et al., 2009). Otherwise the prognoses for most *RAPSN*-CMS patients are good, with the frequencies of episodic worsening reducing with age if appropriate treatments were provided (Milone et al., 2009). Most patients show clear-cut clinical improvements when treated symptomatically with the most commonly used anti-cholinesterase inhibitor, pyridostigmine (Burke et al., 2003; Milone et al., 2009), a drug that acts to prolong the lifetime of acetylcholine at the NMJ (Finlayson et al., 2013). In cases where pyridostigmine fails to show optimal effects, 3,4-diaminopyridine (3,4-DAP), a potassium channel blocker that increases the release of acetylcholine by the presynaptic nerve terminal is given to patients to further sustain neuromuscular transmission (Banwell et al., 2004; Burke et al., 2003; Finlayson et al., 2013; Milone et al., 2009).

1.5.2 AChR deficiency phenotypes in CMS

AChR deficiency was initially reported in CMS due to mutations in *CHRNE* (Ohno et al., 1997), and it is the most common form of CMS in the UK (Finlayson et al., 2013). *CHRNE* mutations can cause AChR deficiency by complete ablation or reduced expression of the ϵ -subunit, or reduce the efficiency of AChR assembly (Ohno et al., 2001a). The γ -subunit is persistently expressed at low levels in the human muscle, and can partially compensate for the impaired neuromuscular transmission due to null mutations in *CHRNE* (Croxen et al., 2001). In rare cases, AChR deficiency due to mutations in *CHRNA1* and *CHRND* has also been reported (Quiram et al., 1999; Shen et al., 2008). The α -, β - and δ - subunits lack a substituting subunit, therefore mutations that completely abolish the expression of these subunits are lethal (Michalk et al., 2008). Mutations in genes encoding AChR subunits can also cause CMS by affecting the kinetics (slow- and fast- channel syndromes) and the conductance of the receptors (Engel et al., 2015; Webster et al., 2012).

RAPSN mutations also result in AChR deficiency phenotype, but the underlying cause for the loss of AChR at the postsynaptic membrane is due to defective clustering and anchoring of the receptors (Cossins et al., 2006; Ohno et al., 2002). Stemming from the differences in the pathogenic mechanisms, clinical features present in *RAPSN*- and *CHRNE*- CMS patients are also distinctly different (Burke et al., 2004; Ohno et al., 1997; Ohno et al., 2002). For example, ophthalmoplegia is a prominent feature in patients with AChR deficiency due to *CHRNE* mutations that does not occur in *RAPSN*-CMS patients; and acute respiratory crises is a characteristic feature in *RAPSN*-CMS that only affects a minority of the most severely affected patients with *CHRNE* mutations (Burke et al., 2004).

1.5.3 Treatments for CMS

Cholinesterase inhibitors, 3,4-DAP, open channel blockers and β_2 -adrenergic agonists are now common medications used for CMS (Engel et al., 2015), and they are usually chosen to target the defective mechanism for specific subtypes.

Pyridostigmine is frequently used for CMS subtypes that reduce the synaptic response to ACh, including *RAPSN*-CMS (Milone et al., 2009), primary AChR deficiency (Engel, 2007) and fast-channel syndromes (Palace et al., 2012). However, patients often respond incompletely to pyridostigmine (Engel, 2007). 3,4-DAP, commonly used as a substitution or in combination with pyridostigmine, is another type of medication that enhances neuromuscular transmission. It blocks outward potassium current at the nerve terminal to prolong calcium ion influx and promote the release of ACh (Kirsch and Narahashi, 1978; Saint, 1989).

Fluoxetine and quinidine are open-channel blockers of AChR that are used for slow-channel syndrome where its underlying defect stems from prolonged opening of the AChR channel pore, causing over stimulation of AChR (Colomer et al., 2006).

Ephedrine and salbutamol are commonly used β_2 -adrenergic agonists. Although their mechanism of action in the NMJ is yet to be defined, the use of these medicines are effective in CMS due to mutations in *COLQ* (Liewluck et al., 2011), *DOK7* (Burke et al., 2013; Lashley et al., 2010), and more recently AChR deficiency due to *CHRNE* mutations as a combination therapy with pyridostigmine (Rodríguez Cruz et al., 2015; Sadeh et al., 2011).

1.6 Objectives

The goal of the presented thesis is to expand current knowledge on the molecular consequences of *RAPSN* mutations so the diagnosis and treatment strategies can be tailored for *RAPSN*-CMS patients. Different aspects of *RAPSN*-CMS will be explored, and the work is divided into 4 parts:

1) Determine the effects of novel *RAPSN* variants on AChR clustering

CMS is a group of rare disorders, and genetic analysis of patient DNA facilitates definitive diagnoses. However, not all gene variants are pathogenic, and it is therefore necessary to perform molecular analyses of newly identified *RAPSN* variants to confirm their pathogenic effects in reducing AChR clustering. The results would build on an existing library of known mutations in *RAPSN* that cause CMS, so that rapid genetic diagnoses can be made in the future.

2) Explore the underlying causes for AChR deficiency in *RAPSN*-CMS

Rapsyn is involved in numerous pathways in the formation and maintenance of AChR clusters. Thus an improved understanding of the molecular consequences of *RAPSN* mutations that lead to AChR deficiency may allow for the development of treatments that specifically target the molecular defects to provide optimal benefits for patients. The underlying cause for the characteristic respiratory crises in *RAPSN*-CMS is not known, and there is currently no available preventative treatment. This study also aimed to explore the presence of a common mechanism through which *RAPSN* mutations might act to induce the acute crises.

- 3) Explore the underlying mechanisms of a mutation in *CHRND* that may attenuate the interaction between rapsyn and AChR

Although it is well known that rapsyn is crucial for the clustering of AChR, the precise points of interaction between rapsyn and AChR at the NMJ is less well understood. A CMS patient with a missense mutation, E381K, and a null allele in *CHRND* had clinical features that are typical of *RAPSN*-CMS. Initial functional analysis revealed that the mutation caused AChR deficiency due to impaired AChR clustering with rapsyn (Muller et al., 2006b). This suggests that the p.E381 residue of the δ -subunit or its nearby regions could be involved in the interactions between rapsyn and AChR, and its subsequent clustering process. Analyses were performed on this mutation to gain further insights.

- 4) A pilot study to explore the beneficial effects of salbutamol in a mouse model of AChR deficiency syndromes

Standard treatments with pyridostigmine or 3,4-DAP do not provide optimal or sustained beneficial effects in most CMS patients with AChR deficiency syndromes (Engel, 2007). Recent reports in literature have suggested that salbutamol could provide further benefits (Rodríguez Cruz et al., 2015; Sadeh et al., 2011), but the mode of action of salbutamol at the NMJ is now known. Hence a pilot study was performed to obtain preliminary data on the efficacy of salbutamol as an additional therapeutic regimen for AChR deficiency syndromes.

Chapter 2

Material and methods

2.1 DNA methods

All chemicals were purchased from Sigma-Aldrich (Sigma). Sanger DNA sequencing of plasmid DNA or PCR products was carried out by the in-house facility provided by the Weatherall Institute of Molecular Medicine (WIMM).

2.1.1 Polymerase Chain Reaction (PCR)

PCR reactions were performed on Veriti thermal cyclers (Applied Biosystems®), using optimised cycling parameters for individual reactions. Oligonucleotides used for PCR analysis were synthesised by Invitrogen.

2.1.1.1 Standard PCR reaction

MegaMix-Blue (Microzone), a PCR master mix that contains recombinant *Taq* polymerase in 1.1x reaction buffer with 2.75mM MgCl₂, 220µM dNTPs, loading dye and stabiliser, was used for standard PCR reactions. Typical reaction mixtures contain 18µl of MegaMix-Blue, 2µl of 10µM primer mix and template DNA.

2.1.1.2 High-fidelity PCR reaction

PfuUltra II Fusion HS DNA polymerase (Agilent Technologies, #600670) was used for high fidelity PCR reactions. This is a thermostable DNA polymerase that allows efficient long target-length amplification with high accuracy. A typical 50µl PCR reaction mix contained 40.5µl of distilled water, 5µl of 10x *PfuUltra* II reaction

buffer, 0.5µl of dNTP mix (25mM of each dNTP), 1µl of DNA template (100ng/µl), 2µl of primer mix (10µM each) and 1µl of *PFuUltra* II Fusion HS DNA polymerase. The cycling parameters for PCR using *PFuUltra* II Fusion HS DNA polymerase is shown in Table 2-1 together with the parameters for using MegaMix-Blue.

Table 2-1 Parameters used for typical PCR reactions

Number of cycles	Temperature	Duration
		MegaMix-Blue/PfuUltra II fusion
STEP 1		
1	95°C	5 minutes/ 2 minutes
STEP 2		
30	95°C	30 seconds/20 seconds
	T _M -5°C	30 seconds/20 seconds
	72°C	1 minute per kb/30 seconds per kb
STEP 3		
1	72°C	10 minutes/ 3minutes
1	4°C	∞

2.1.1.3 Resolution of DNA on agarose gels

Agarose gel electrophoresis was used to resolve DNA fragments. The standard percentage of agarose used was 1% dissolved in 1x TBE buffer (90mM Trizma base, 90mM boric acid and 2mM EDTA) supplemented with ethidium bromide (0.5µg/ml) to visualise nuclei acids. Gels made with 2% agarose were used to separate smaller DNA fragments. DNA samples were prepared in a mixture with a loading buffer (4mM bormophenol blue, 0.35M EDTA, 30% glycerol) and ran in 1x TBE at 60-100 V and analysed using a UV transiluminator. If necessary, DNA bands in agarose gels were excised and purified using the Qiaquick gel extraction kit (QIAGEN, 28703) following the manufacturer's protocol.

2.1.2 Basic Molecular cloning

Restriction enzymes were purchased from New England Biolabs (NEB) and used according to the manufacturer's protocol. To prevent re-annealing of vector DNA after restriction digestion by the use of a single enzyme, linearised plasmid DNA was dephosphorylated using Antarctic phosphatase (NEB, M0289). DNA ligations were performed using T4 DNA ligase (Invitrogen, 15224) in a reaction mixture, recommended by the manufacturer, at 16°C for 16 hours in a reaction mixture. Typically, a 5:1 molar ratio of insert to vector was used for ligation reactions.

2.1.2.1 Bacterial Transformation

Chemically competent bacterial cells, *Escherichia coli* (*E. coli*) strains JM109 (NEB), XL10-Gold (Stratagene), and TOP10 (Invitrogen) were used to replicate plasmid DNA. The competent cells were cultured in lysogeny broth (LB) medium (10g/L of NaCl, 10g/L of tryptone, 5g/L of yeast) or LB-agar plates (15g of agar/L of LB).

Between 10ng to 100ng of DNA were transformed into the bacterial strains thawed on ice by heat shock methods (Table 2-2). 4µl of β-mercaptoethanol was added XL10-Gold cells and was incubated in ice for 10 minutes before DNA was added.

Table 2-2 Transformation conditions for competent bacterial cells

	JM109	XL10-Gold	TOP10
ice	10 minutes	30 minutes	30 minutes
heat shock	42°C for 30 sec	42°C for 45 sec	42°C for 30 sec
ice	2 minutes	2 minutes	2 minutes

Competent cells were initially incubated in ice, followed by heat shock and then back in ice for 2 minutes

After the heat shock process, 400µl of LB were added to the transformation mixtures and the cells were incubated at 37°C for 1 hour in a shaking incubator and then plated onto LB-agar plates supplemented with appropriate antibiotics and incubated at 37°C overnight. The concentrations of antibiotics used are summarised in Table 2-3.

Table 2-3 Antibiotic concentrations used to select transformed bacterial cells

Antibiotic	Concentration in LB/LB-agar
Ampicillin	100µg/ml
Kanamycin	50µg/ml
Ampicillin, IPTG, X-GAL	100µg/ml, 0.5mM, 80µg/ml

Competent cells transformed with pBabe-PURO-sn, pcDNA3.1 and pRc/CMV2 plasmids were selected with ampicillin; pEGFP-N1 with Kanamycin; and pGEM-T Easy vectors with a mixture of ampicillin, IPTG (isopropyl thiogalactoside) and X-GAL (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside)
All of the antibiotics were purchased from Sigma.

Plasmid DNA extracted from bacterial cultures was purified using the QIAprep spin miniprep kit (QIAGEN, 27106) or the PureLink HiPure plasmid filter maxiprep kit (Invitrogen, K2100-17) according to the protocols from the manufacturers.

2.1.2.2 pGEM-T Easy cloning

PCR products were cloned into pGEM-T Easy vectors according to the protocol provided by the manufacturer (Promega, A1380). The pGEM-T Easy vector supplied is a linearised plasmid that possesses a 3' terminal thymidine overhang at both ends. Thus blunt-ended PCR products were subjected to an A-tailing procedure prior to the ligation into the vector at the 5:1 molar ratio. Typical reactions set up for A-tailing and insert ligations are shown in Table 2-4.

Table 2-4 Reaction mixtures for pGEM-T-Easy cloning

Poly-A tailing at 70°C for 30 minutes	
DNA (PCR product)	7µl
Taq Buffer (10x)	1µl
dATP (2nM)	1µl
MgCl ₂ (50mM)	0.333µl
Taq polymerase	1µl
Ligation of insert into pGEMT-Easy vector at 16°C for 16 hours	
pGEMT-Easy vector (50ng)	1µl
Insert (5:1 molar ratio to vector)	
2x ligation buffer	10µl
T4 ligase	1µl
ddH ₂ O	up to 20µl total volume
T4 ligase used in pGEMT-Easy cloning was supplied with the vector	

Reaction mixtures that contained the ligated plasmids (2µl) were transformed into JM109 competent cells. The transformed cells were plated on LB-agar plates supplemented with ampicillin, IPTG and X-Gal and cultured at 37°C overnight. Subsequently, colonies were picked and analysed.

2.1.3 Plasmids available in the laboratory

A list of the plasmids used in this thesis is shown in Table 2-5 with a brief description on their functions. Site-directed mutagenesis and molecular cloning performed on selected plasmids are described in subsequent sections.

Table 2-5 DNA plasmids that were available in the laboratory and used for experiments

Plasmid	Description
pBabe- <i>Chrnd</i> -IRES-EGFP-PURO-sn	Retroviral plasmid for the expression of mouse AChR δ -subunit with an internal ribosome entry site so that the fluorescent marker enhanced green fluorescent protein (EGFP) is also expressed but not tagged to the subunit
pBabe- <i>RAPSN</i> -EGFP-PURO-sn	Retroviral plasmid for the expression of human rapsyn-EGFP
pcDNA3.1- <i>AGRN</i>	Expression plasmid that encodes full-length human agrin
pcDNA3.1-rat <i>Agrn</i>	Expression plasmid that encodes C-terminal of rat agrin
pcDNA3.1- <i>mCherry</i>	Expression plasmid that encodes the fluorescent marker mCherry
pEGFP-N1	Expression plasmid that encodes the fluorescent marker enhanced green fluorescent protein (EGFP)
pEGFP-N1- <i>RAPSN</i>	Expression plasmid that encodes human rapsyn-EGFP
pRC/CMV2- <i>Chrna1</i>	Gifted by Dr. J Muller (Muller et al., 2006) Expression plasmids that encode the mouse AChR subunits: α, β, δ, ϵ respectively
pRC/CMV2- <i>Chrnb1</i>	
pRC/CMV2- <i>Chrnd</i>	
pRC/CMV2- <i>Chrne</i>	
pX335-U6-Chimeric_BB-Cbh-hSPCas9n(D10A)	Gifted by Dr Joey Riepsaame (WIMM) and the plasmid is referred to as px335-Cas9n in text The plasmid encodes the Cas9 nickase mutant D10A. Guide oligonucleotide sequences were ligated into the sgRNA scaffold of the plasmid as part of the protocol for the CRISPR/Cas9n mutagenesis system used

2.1.4 Site-directed mutagenesis

Oligonucleotides used in site-directed mutagenesis were synthesised by Invitrogen.

The QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene, 200515) was used to generate single base substitutions in the pBabe-*RAPSN-EGFP*-PURO-sn, pEGFP-N1-*RAPSN* and pBabe-*Chrnd*-IRES-*EGFP*-PURO-sn plasmids. For each mutation, a specifically designed mutagenic primer, with the desired mutation located centrally, was used in the thermal cycling reaction with *PFuTurbo* DNA polymerase to generate plasmid DNA with one strand that harbours the mutation. Subsequently, DpnI, an endonuclease that is specific for methylated and hemimethylated DNA was used to digest the DNA template. This is because dam^+/dcm^+ *E. coli* strains were used to generate the original template plasmids, causing the templates to become methylated and susceptible to DpnI digestion. The DNA was transformed into XL10-Gold or TOP10 competent cells and the transformed cells were cultured on LB-agar containing appropriate selection antibiotics.

Following a similar protocol, the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, 200521) was used to generate multiple base deletions in the pRC/CMV2-*Chrnd* plasmid. This method required a pair of mutagenic primers to synthesise double-stranded mutant plasmid DNA with *PFuUltra* DNA polymerase. Tables 2-6 and 2-7 show the list of oligonucleotide mutagenic primers used.

For both protocols, successful mutagenesis reactions were confirmed by the sequencing of plasmids purified from antibiotic-resistant colonies using the primers listed in Tables 2-8 and 2-9.

Table 2-6 Sequences of primers used for mutagenesis in *RAPSN* cDNA

<i>RAPSN</i> mutations	Primer name	Primer sequence (5' to 3')
p.E23K	RAPSN_mut.E23K_F	CCAGTCCAACCAGACAAAGAAGGCATTGCAGG TG
p.V45M	RAPSN_mut.V45M_F	CTCATGGGGCGCTTCCGCATGCTGGGCTGCCT GGTCACAG
p.R74Q	RAPSN_mut.R74Q_F	CAGATCGACACGGCCCAGGAGCTGGAGGATGC CGAC
p.N88K	RAPSN_mut.N88K_F	CCTCCTGGAGAGCTACCTGAAACTGGCACGCA GCAACGAGAAGC
p.R91L	RAPSN_mut.R91L_F	GCTACCTGAACCTGGCACTCAGCAACGAGAAG CTGTGC
p.A153T	RAPSN_mut.A153T_F	GAAGGCCCTGCGCTATACCCACAACAATGA
p.S201N	RAPSN_mut.S201N_F	CTATGGCAAAGGCTGGAACCTGAAGTACCGGG CCATG
p.R257W	RAPSN_mut.R257W_F	GCTTCGCTGACATCCACTGGAGCCGTGGGGAC CTGG
p.T264I	RAPSN_mut.T264I_F	GCCGTGGGGACCTGGAGATAGCCTTCCCCAGG TACG
p.S274N	RAPSN_mut.S274N_F	AGGTACGACTCCGCCATGAACATCATGACCGA GATCG
p.H329P	RAPSN_mut.H329P_F	GCTGAGCCAGCTCAAGCTGCCCTGTCTGAGCG AGAG
p.R376W	RAPSN_mut.R376W_F	GGCGAGAAGAACAGCTGGCTGCAGGCCCTACC TTGC
p.H387Y	RAPSN_mut.H387Y_F	CCCACATCTTCTACCTCAGGTGCCTGCAGAAC AACG
p.C399S	RAPSN_mut.C399S_F	GGGACCCGGAGCAGTCCCAACTGCCGCCGCTC ATCC

Table 2-7 Sequences of primers used for mutagenesis in *Chrnd* cDNA

<i>Chrnd</i> mutations	Primer name	Primer sequence (5' to 3')
p.E381K	Chrnd_mut.E381K_F	GCAGTGACCTCATGTTTAAGAAGCAATCAGAGCG GC
c.del1136_1148	KO6_mut.v1_F	CCCGCAGTGACCTCAATCAGAGCGGCATGG
	KO6_mut.v1_R	CCATGCCGCTCTGATTGAGGTCAGTGC
c.del1132_1156	KO6_mut.v2_F	CTCACTTAAGTCCCGCAGTGACGGCATGGGCTGG CCCGGCGC
	KO6_mut.v2_R	GCGCCGGGCGCAGCCCATGCCGTCACTGCGGGACT TAAGTGAG
c.del1131_1143	KO6_mut.v3_F	CTCACTTAAGTCCCGCAGTGAAAGCAATCAGAGC GGCATGGG
	KO6_mut.v3_R	CCCATGCCGCTCTGATTGCTTTCACTGCGGGACT TAAGTGAG

Table 2-8 Sequencing primers used to confirm the sequences of *RAPSN* cDNA

Primer name	Primer sequence (5' to 3')
RAPSN1_cDNA_seqF	AAGTTCGCTGTGGTCCAGAT
RAPSN2_cDNA_seqF	CAATGCCTTCCTGGGCCTCA
RAPSN3_cDNA_seqF	GCAAAGGCTGGAGCCTGAAG
RAPSN4_cDNA_seqF	ACAGCCTTCCCCAGGTACGA
RAPSN5_cDNA_seqF	TCTGAGCGAGAGCATTTACC
PBABE_5'_seqF	CTTTATCCAGCCCTCAC
PBABE_3'_seqR	CTAACTGACACACATTCCAC

Table 2-9 Sequencing primers used to confirm the sequence of *Chrnd* cDNA

Primer name	Primer sequence (5' to 3')
Chrnd_p.E381KcDNA_seqF	CCCTGCCCCAAGCTCCTGCAC
Chrnd_p.E381KcDNA_seqR	TTTGCCCCATCCACAGCTGG

2.1.5 Specific cloning

To evaluate the feasibility of applying the Halo- or SNAP- tag systems to follow the expression and localisation of rapsyn, the genes of the protein tags were cloned into pBabe-*RAPSN*-EGFP-PURO-sn to replace *EGFP*.

2.1.5.1 Cloning of HaloTag into pBabe-*RAPSN*-PURO-sn

Two BamHI restriction sites in the plasmid pBabe-*RAPSN*-EGFP-PURO-sn were used to replace the EGFP gene with the gene encoding Halo-tag (HaloTag). The strategy used was to amplify the HaloTag from the pHTC-HaloTag vector (Promega, G7711) by PCR. The manufacturer's protocol suggested that it is critical to also include the HaloTag-linker and the TEV protease recognition sequence to ensure optimal function of the HaloTag coding region. However, a BamHI restriction recognition site was present within the region of interest (Figure 2-1).

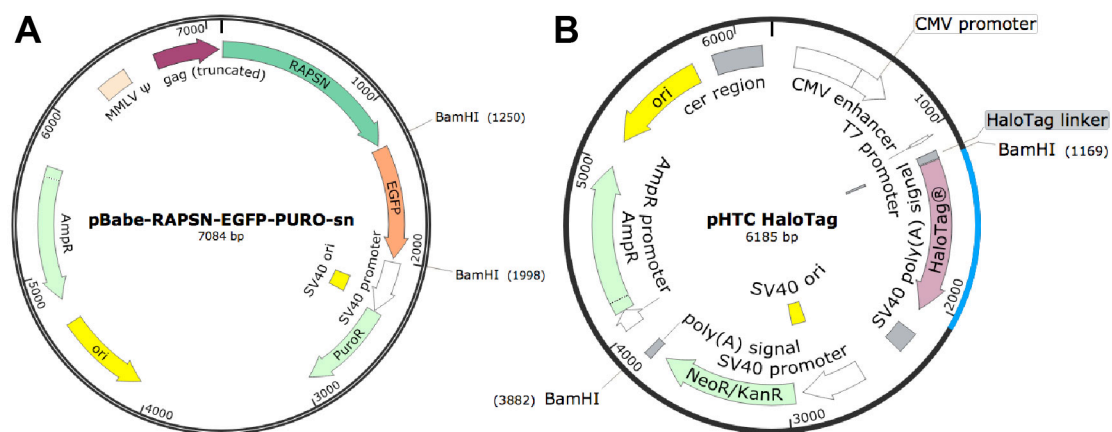


Figure 2-1 Schematic diagrams of pBabe-*RAPSN*-EGFP-PURO-sn and pHTC-HaloTag

The figure shows the BamHI restriction recognition sites on both plasmids. The BamHI site at the region of interest (**B**, blue of the backbone) in pHTC-HaloTag was subsequently removed by the introduction of a silent mutation at the restriction recognition site.

The BamHI site is located at the first codon of the HaloTag, and was eliminated by using the QuikChange Multi Site-Directed Mutagenesis Kit and a mutagenic primer, 5'-CAGAGCGATAACGATGGGTCCGAAATCG-3', to introduce a silent mutation GGA>GGC within the restriction recognition sequence.

The HaloTag gene and associated linker (964bp) from the pHTC-HaloTag plasmid with the silent mutation was amplified using *PfuUltra* II Fusion HS DNA polymerase and a pair of primers with overhangs that correspond to BamHI recognition sequences at their 5' ends (Table 2-10). An additional base was added between the BamHI and HaloTag-linker sequences so that the HaloTag is in frame with *RAPSN* when ligated into pBabe-*RAPSN-EGFP*-PURO-sn.

Table 2-10 Primers used to amplify the HaloTag gene with the HaloTaglinker

Target gene	Primer name	Primer sequence (5' to 3')
HaloTag-linker and gene with BamHI overhangs	BamHI_Link_HaloTag F	GTACGGGATCCACTCGAGCCAACCACTGAG
	BamHI_Link_HaloTag R	GCATGGGGATCCTTAACCGGAAATCTC

The PCR products were initially cloned into pGEM-T Easy vectors. The ligation products and pBabe-*RAPSN-EGFP*-PURO-sn were digested with BamHI and analysed by gel electrophoresis. The correct bands were excised and purified for subsequent ligation of the HaloTag into pBabe-*RAPSN-EGFP*-PURO-sn. The reaction products were transformed into TOP10 competent cells and purified. The correct orientation of HaloTag in the plasmid was confirmed by PCR analysis using the forward primer localised within the *RAPSN* gene and reverse primer in HaloTag

(Table 2-11). The PCR products were resolved and the correct plasmids produced a band at approximately 911bp. The correct sequences of the pBabe-*RAPSN*-HaloTag-PURO-sn plasmid (Figure 2-2) was confirmed by DNA sequencing.

Table 2-11 Primers used to determine the orientation of the HaloTag gene in pBabe-*RAPSN*-HaloTag-PURO-sn

Primer name	Primer sequence (5' to 3')
RAPSN5_cDNA_seqF	TCTGAGCGAGAGCATTTACC
Halo_orientation_PCR_R	GCCACAGTGGCTCGCGGTCA

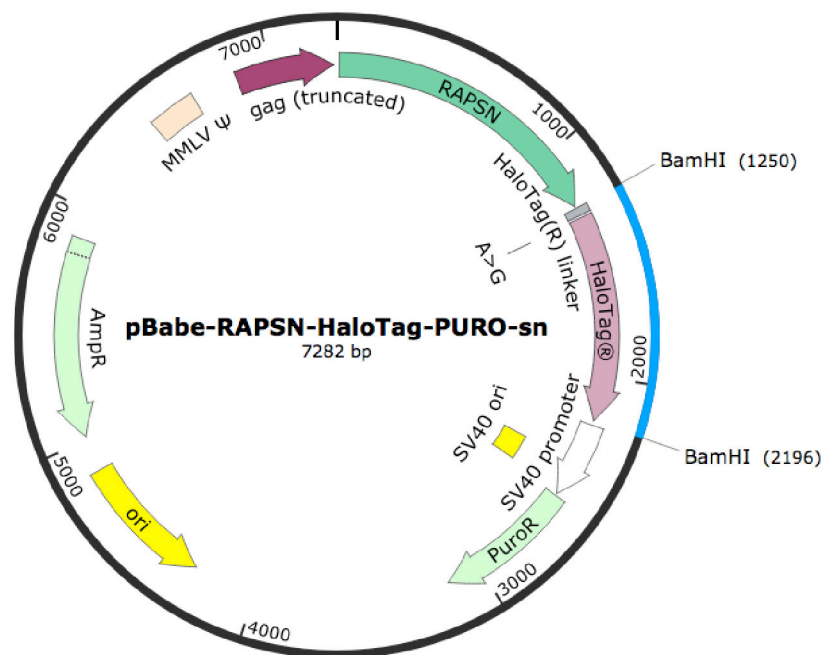


Figure 2-2 Schematic diagram of pBabe-*RAPSN*-HaloTag-PURO-sn

The HaloTag gene and the HaloTag-linker (highlighted in blue on the backbone) were cloned into pBabe-*RAPSN*-EGFP-PURO-sn to replace the *EGFP* via the BamHI restriction sites. A silent mutation was introduced to the first codon of the HaloTag gene to remove an unwanted BamHI restriction recognition site before the region of interest was amplified by PCR and subsequent procedures.

2.1.5.2 Cloning of SNAPTag into pBabe-RAPSN-PURO-sn

A similar approach was used to replace *EGFP* from pBabe-*RAPSN-EGFP*-PURO-sn with the gene encoding SNAP-tag (*SNAP_f*). *SNAP_f* was PCR amplified from pSNAPf vector (NEB, N9183S) using *PFuUltra* II Fusion HS DNA polymerase and the primers listed in Table 2-12.

Table 2-12 Primers used to amplify SNAP_f

Target gene	Primer name	Primer sequence (5' to 3')
<i>SNAP_f</i> with BamHI overhangs	BamHI_Linkers_SNAP F	TGTACGGGATCCAATGGACAAAGACTGCGAAATG
	BamHI_Linkers_SNAP R	GCATGGGGATCCCTAACCCAGCCCAGGCTTGCC

SNAP_f was first ligated into the pGEM-T Easy vector, digested with BamHI and then cloned into pBabe-*RAPSN-EGFP*-PURO-sn via its BamHI restriction digest sites. The orientation of *SNAP_f* in the plasmid was confirmed by PCR analysis using the pair of primers listed in Table 2-13. The final product, pBabe-*RAPSN-SNAP_f*-PURO-sn (Figure 2-3) was confirmed to be correct by DNA sequencing.

Table 2-13 Primers used to amplify SNAP_f

Primer name	Primer sequence (5' to 3')
RAPSN3_cDNA_seqF	GCAAAGGCTGGAGCCTGAAG
SNAP_orientation_PCR_R	GGCGGTGGCCTGCATCAGTG

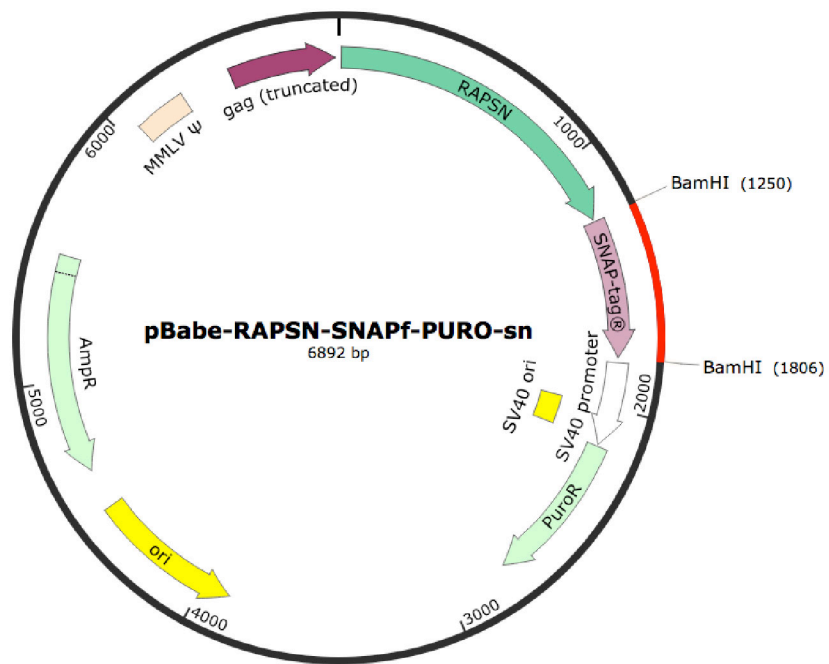


Figure 2-3 Schematic diagram of pBabe-RAPSN- SNAP_f -PURO-sn

SNAP_f (red) was cloned into pBabe-RAPSN-EGFP-PURO-sn to replace the EGFP via the BamHI restriction sites.

2.2 Cell culture methods

The cell lines used in this thesis were cultured as described in Table 2-14 and (Cossins et al., 2006; Cossins et al., 2012; Muller et al., 2006b) in Corning tissue culture plasticware, or in FluoroDish glass-bottom dishes (World Precision Instruments, FD35-100) for confocal microscopy. C2C12 and *Rapsn*-knockout (KO) (Fuhrer et al., 1999) cells were differentiated into myotubes, by the change of culture medium and conditions upon reaching 100% confluency. The cells were differentiated on surfaces that were uncoated or pre-coated with BD Matrigel matrix (VWR, 734-0272) respectively. The differentiation medium was refreshed every other day until mature myotubes were formed.

Table 2-14 Conditions for cell culture

Growth conditions		
Cell lines	Dulbecco's modified Eagle medium (DMEM) supplemented with	Conditions
C2C12	15% FCS + 1% antibiotics	37°C, 8% CO ₂
GP +E86	10% FCS + 1% antibiotics	37°C, 8% CO ₂
HEK 293	10% FCS + 1% antibiotics	37°C, 8% CO ₂
<i>Rapsn</i> -knockout	20% FCS + Interferon- γ (0.2 μ g/ ml) + 0.5% chick embryo extracts + 1% antibiotics	33°C, 5% CO ₂
TE671	10% FCS + 1% antibiotics	37°C, 8% CO ₂
Differentiation conditions		
Cell lines	Dulbecco's modified Eagle medium (DMEM) supplemented with	Conditions
C2C12	2% FCS + 1% antibiotics	37°C, 8% CO ₂
C2C12 clonal cell lines treated with CRISPR-Cas9n	2% FCS + 10% HS + 1% antibiotics	37°C, 8% CO ₂
<i>Rapsn</i> -knockout	10% HS + 1% antibiotics	37°C, 8% CO ₂

Antibiotics = penicillin, streptomycin and amphotericin B (Invitrogen, 15240-026), chick embryo extract (GENTAUR, CE-650-T), DMEM (Sigma, D6429); FCS = foetal calf serum (Invitrogen, 10270), HS = horse serum (Sigma, H1138); Interferon- γ (Sigma, I4777)

2.2.1 Transfection and retroviral infection methods

2.2.1.1 Polyethylenimine (PEI) transfections

HEK 293 and GPE +86 were transiently transfected with DNA plasmids using PEI (45mg PEI in 8ml distilled water, pH 7.2) in transfection mixtures as stated in Table 2-15. Sixteen hours post-transfection, the transfection mixtures were replaced by fresh culture medium and the cells were cultured until subsequent procedures.

Table 2-15 Polyethylenimine (PEI) transfections

Cell line	Seeding density	Transfection mixture					
		Plasmid	Amount	20% Glucose	PEI	H ₂ O	Culture medium
GPE +86	6x10 ⁵ per T25 flask	pBabe- <i>Chrnd</i> -IRES-EGFP-PURO-sn	16µg	6.6µl	3.75µl	up to 40µl	2.5ml
GPE +86	6x10 ⁵ per T25 flask	pBabe- <i>RAPSN</i> -EGFP-PURO-sn	16µg	6.6µl	3.75µl	up to 40µl	2.5ml
HEK 293	3x10 ⁵ per well of 6-well plate	*pRC/CMV2- <i>Chrna1/Chrb1/Chrnd/Chrne</i>	3µg in total with the ratio of 2α:1β:1δ:1ε	1.25µl	1.5µl	up to 7.5µl	1ml
HEK 293	2x10 ⁶ per T75 flask	pcDNA3.1- <i>AGRN</i>	18µg	7.5µl	9µl	up to 45µl	6ml
HEK 293	2x10 ⁶ per T75 flask	pcDNA3.1-rat- <i>Agrn</i>	18µg	7.5µl	9µl	up to 45µl	6ml
HEK 293	3x10 ⁵ per well of 6-well plate	#pEGFP-N1- <i>RAPSN</i>	3µg	1.25µl	1.5µl	up to 7.5µl	1ml

0.5µg of *pEGFP-N1 or #pcDNA3.1-mCherry was added to the transfection mixture to determine transfection efficiency.

2.2.1.2 Electroporation

TE671 and C2C12 cells were electroporated with DNA using the Neon transfection system (Invitrogen, MPK5000). Prior to electroporation, the cells were centrifuged at 1200rpm for 5 minutes and washed with PBS, centrifuged again and resuspended in 125µl of resuspension buffer R containing the DNA to be transfected into cells. 100µl of the mixture were transferred to the Neon tube filled with 3ml of electrolytic buffer E2 that is attached to the Neon electroporator. Once electroporated, the cells were transferred to pre-warmed growth medium without antibiotics in 6-well plates and T25 tissue culture flasks for TE671 and C2C12 cells respectively. 24 hours post-transfection, cells were cultured in normal culture medium containing antibiotics. The parameters used are summarised in Table 2-16.

Table 2-16 Parameters for Neon-transfections

Cell line	Cell density per 100µl	DNA	Amount	Pulse voltage (v)	Pulse width (ms)	Pulse number
TE671	5x10 ⁵	*pEGFP-N1-RAPSN	3µg	1100	30	1
¹ C2C12	5x10 ⁵	pX335-guideA-guideB-Cas9n Repair template (10µM)	10µg 10µl	1650	10	3
² C2C12	5x10 ⁵	pX335-guideA-guideB-Cas9n Repair template (10µM)	20µg 10µl	1650	10	3
³ C2C12	5x10 ⁵	pX335-guideA-guideB-Cas9n	10µg	1650	10	3

* The cells were co-electroporated with 0.5µg of pcDNA3.1-mCherry as the control for transfection efficiency. Mutant cells A11, B15 and KO6 were generated by the electroporations marked with ¹, ² and ³ respectively.

2.2.1.3 Generation of recombinant agrin

HEK 293 cells were transfected with pcDNA3.1-*AGRN* or pcDNA3.1-rat-*Aggrn*, which encodes for full-length human agrin and C-terminal rat agrin by PEI transfection. Sixteen hours post transfection, the culture medium was replaced by 6ml of fresh medium. The cells were cultured for a further 72 hours, and the medium containing soluble agrin was harvested and centrifuged at 2000rpm for 10 minutes. The supernatant was kept and stored at -20°C.

2.2.1.4 pBabe retroviral system

To produce retroviruses, variants of pBabe-*RAPSN-EGFP*-PURO-sn, pBabe-*RAPSN*-HaloTag-PURO-sn, pBabe-*RAPSN-SNAPγ*-PURO-sn or pBabe-*Chrnd*-IRES-*EGFP*-PURO-sn were transfected into the retroviral packaging cell line GP +E86 (Markowitz et al., 1988) by PEI transfection. Two days post-transfection, puromycin (Sigma, P8833) was introduced to the culture medium of the cells at 5µg/ml to select for stable transfectants, which once repopulated were seeded at 2.5×10^6 cells in T75 flasks. The cells were incubated in 6ml of medium without puromycin for 72 hours. The medium containing the retroviruses was then harvested, centrifuged at 20000rpm for 10 minutes to remove debris and frozen for storage at -80°C.

To achieve stable expression of rapsyn-EGFP or exogenous AChR δ -subunit, *Rapsn*-KO or C2C12 myoblasts were infected with retroviruses. 5×10^4 cells were seeded in T25 culture flasks and incubated with 1ml of the medium containing retroviruses for 16 hours on the following day. After retroviral infection, the cells were cultured in normal growth medium, and puromycin selection (5µg/ml) began 2 days later. The stably infected cells were grown in growth medium supplemented with puromycin.

2.3 CRISPR-Cas9n system

The CRISPR-Cas9n system was used to introduce the E381K mutation or truncating deletions in proximity to p.E381 of *Chrnd* in the C2C12 cells. This was performed according to the protocol stated in (Ran et al., 2013b) with slight adaptations. The principles of the methodology used are described in Chapter 5.

2.3.1 Preparation of the CRISPR-Cas9n construct

The pX335-U6-Chimeric_BB-Cbh-hSPCas9n(D10A) plasmid (pX335-Cas9n) was provided by Dr Joey Riepsamme. A pair of guide sequences (guide A and guide B, both 20 base pairs in length) that are complementary to sequences close to p.E381 in *Chrnd* were separately cloned into the pX335-Cas9n vector via a BbsI restriction site. In the vector, the guide sequences are immediately followed by the sgRNA, together referred to as the CRISPR RNA (crRNA), which is required by the system.

The guide sequences (guide A and guide B) were designed using the CRISPR Design Tool (<http://crispr.mit.edu>), and overhangs were added to the sequences so that the guides can be ligated into the BbsI digested plasmids (Table 2-17).

Table 2-17 Oligonucleotide sequences of guide A and guide B used in the CRISPR-Cas9n system

Single-stranded oligonucleotide		Sequence (5' to 3')
Guide A	Top-strand	CACCGCTCAAACATGAGGTCAGTGC
	Bottom-strand	AAACGCAGTGACCTCATGTTTGAGC
Guide B	Top-strand	CACCGCAATCAGAGCGGCATGGGC
	Bottom-strand	AAACGCCCATGCCGCTCTGATTGC

The single-stranded oligonucleotides (Integrated DNA Technologies-IDT) were phosphorylated and annealed according to parameters stated in Table 2-18.

Table 2-18 Parameters for annealing oligonucleotides

Reaction mixture		Parameters used on the thermocycler	
Component	Amount (μl)		
Top-strand (100μM)	1	37°C	30 minutes
Bottom-strand (100μM)	1	95°C	5 minutes
T4 ligation buffer, 10x	1		
T4 polynucleotide kinase (NEB)	1	Ramp down to 25°C at 5°C/minute	
ddH ₂ O	6		

To clone the annealed oligonucleotides into pX335-Cas9n, the plasmid was digested with BbsI, and ligation reactions using T4 ligase were set up for guide A and guide B individually. 2μl of the ligation products were transformed into TOP10 competent cells and the cells were cultured on LB-agar plates supplemented with ampicillin. DNA from 5 colonies was extracted and sequenced to confirm the successful generations of pX335-guideA-Cas9n and pX335-guideB-Cas9n with the U6 Forward primer: 5'-GAGGGCCTATTT CCCATGATTCC-3'.

The next step was to clone the sgRNA scaffold of guide B into pX335-guideA-Cas9n via the restriction sites XbaI and KpnI that are located downstream from guide A. The sgRNA scaffold from pX335-guideB-Cas9n was PCR amplified with primers that possess the recognition sequences of XbaI and KpnI as overhangs at their 5' ends (Table 2-19). The availability of 2 restriction sites on the vector ensures the crRNA scaffold of guide B will be inserted in the correct orientation.

Table 2-19 Primers used to amplify the sgRNA cassette pX335-guideB-Cas9n

Primer	Sequence (5' to 3')
GuidePCR F	GCCATCTAGAGAGGGCCTATTTCCCATGAT
GuidePCR R	GCCAGGTACCGTCTGCAGAATTGGCGCACG

The amplicons of crRNA from pX335-guideB-Cas9n were cloned into pGEM-T Easy, and the ligated vector and pX335-guideA-Cas9n were digested using restriction enzymes XbaI and KpnI. Reaction products were resolved by gel electrophoresis and appropriate bands were excised and gel purified for subsequent ligation reactions at 5:1 molar ratio of insert to vector using T4 ligase. After transformation into TOP10 competent cells and subsequent purifications, the sequences of guide A and guide B in pX335-guideA-guideB-Cas9n were confirmed to be correct by DNA sequencing using the primers listed in Table 2-20.

Table 2-20 Sequencing primers used to confirm the sequences of sgRNA cassettes in pX335-guideA-guideB-Cas9n

Primer	Sequence (5' to 3')
GuideA_seq F	TTGCTGGCCTTTTGCTCACATGT
GuideB_seq R	GTAACGGGTACCGTCTGCAG

A schematic representation of pX335-guideA-guideB-Cas9n is shown in Figure 2-4.

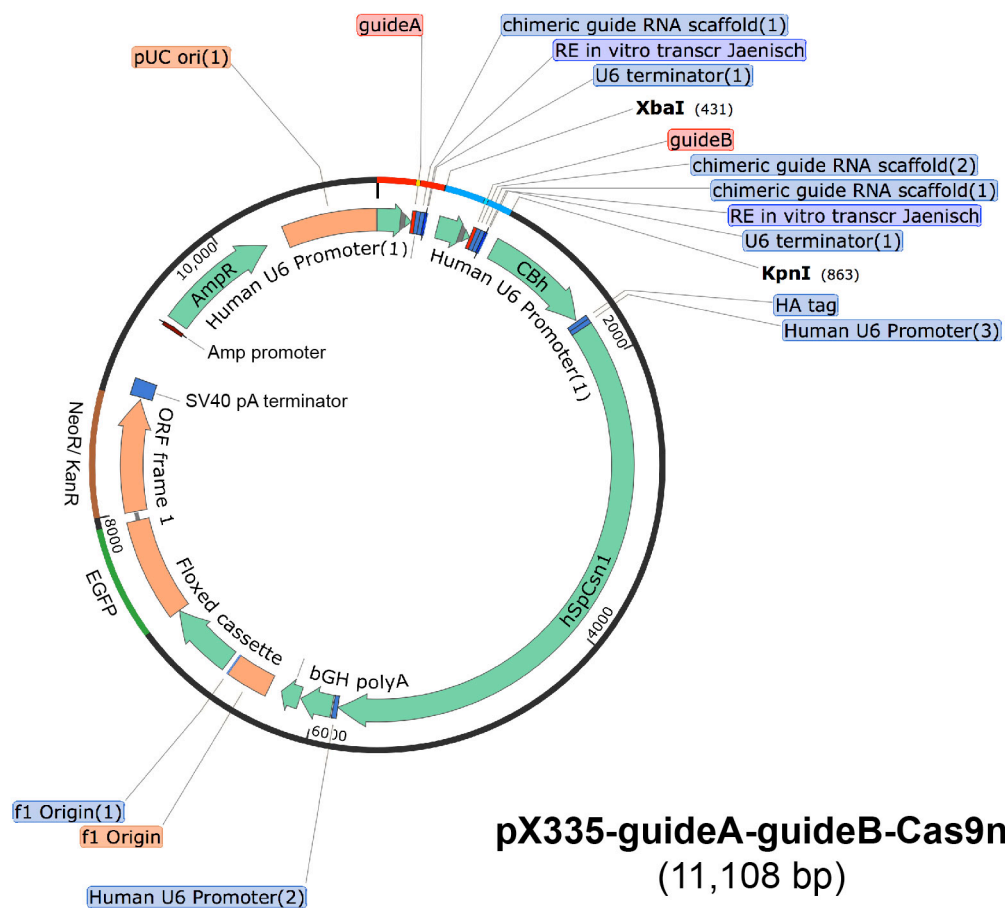


Figure 2-4 Schematic diagram of pX335-guideA-guideB-Cas9n

The guide A and guide B sequences were inserted into their respective sgRNA scaffold (highlighted in red and blue) in the pX335-guideA-guideB-Cas9n plasmid. The plasmid also contains genes that encode for EGFP and neomycin-resistance to allow for selection of cells that were transfected with this plasmid.

2.3.2 Generation of control clonal C2C12 cell lines for the CRISPR-Cas9n system

To generate control cells that were used to evaluate the properties of mutant C2C12 cell lines, the pX335-Cas9n plasmid, which lacks the guide sequences to direct Cas9n to target sites, was electroporated into C2C12 myoblasts. Twenty-four hours after electroporation, the cells were incubated with G418 (Geneticin, Invitrogen) at an active dose of 1mg/ml in culture medium for a further 48 hours to select transfected cells. Subsequently, clonal cells were isolated by limiting dilution at 0.25 cells per well of 2 96-well plates. The clonal colonies were expanded, and DNA from approximately 5×10^4 cells was extracted using the 50µl of QuickExtract solution (Epibio, QE09050) according to the manufacturer's instructions. 2µl of the extracted DNA was analysed by PCR using the MegaMix-Blue master mix and primers that were designed to amplify a genomic region that covers p.E381 in *Chrnd* (Table 2-21). PCR products were resolved on electrophoresis gels and were expected to be 745bp in size. The correct bands were gel purified and analysed by DNA sequencing using primers in Table 2-21, to confirm their wild-type status.

Table 2-21 PCR Primers used to detect mutations within the target region of CRISPR-Cas9n in *Chrnd* of C2C12 cells

Primer	Sequence (5' to 3')
ChrndE381K_screen F	CAGTGGGGAGTCTGGGAAAC
ChrndE381K_screen R	GTGTACCCCTGAGCTTGGTC

2.3.3 Generation of clonal C2C12 cell lines using the CRISPR-Cas9n system

To introduce truncating deletions or the specific E381K mutation in *Chrnd* in C2C12 cells, the pX335-guideA-guideB-Cas9n plasmid was, respectively, electroporated alone or together with a repair template into myoblasts.

The lack of a repair template allows non-homologous end joining of DNA that has been double nicked by Cas9n to generate random indels at the target region. This strategy was used to introduce frameshifts and truncating mutations in *Chrnd* of cells.

On the other hand, a repair template was used to promote homology-directed repair to introduce the E381K mutation to the genome of C2C12 cells (Table 2-22). The repair template is a single-stranded oligonucleotide (123bp in length, IDT) with the mutation GAG>AAG for E381K located centrally. In addition, the 5'-NGG protospacer adjacent motifs (PAM) on the genomic DNA that are immediately downstream to the sequences complementary to guide A and guide B were silently mutated on the template.

Table 2-22 Sequence of the single-stranded oligonucleotide repair template for E381K

Single-stranded oligonucleotide	Sequence (5' to 3')
Repair template for <i>Chrnd</i> _E381K	GGATACATCTGCAAAGCAGAGGAGTATTTCTCACTTAAGTCTCGCAGTGACCT CATGTTTAAGAAGCAATCAGAGCGGCATGGGCTAGCCCGGCGCCTCACCACAG CCCGTGAGTTCTGGTAG

Twenty-four hours after electroporation, the cells were selected using G418 and clonal cells were isolated as described in 2.3.2. For each condition, 5 plates of cells were seeded and allowed to repopulate, which usually requires 2-3 weeks. The repopulated cells were expanded in 24-well plates and approximately a fourth of the cells (at 70% confluency) were subjected to genotype analysis. The remainder were expanded in 6-well plates and passaged. Only cells that possess the desired genotypes were further expanded and used in subsequent experiments.

DNA from cells harvested for genotyping were extracted and analysed as described in 2.3.2. For cells that were purposed to harbour truncating deletions, purified PCR products were sequenced to detect mutations using the same primers used to amplify the region of interest (Table 2-21). For cells that were electroporated with a repair template, the silent mutation at the second PAM site (PAM2) gives rise to an *NheI* restriction recognition site, and purified PCR products were digested using the restriction enzyme. Successful restriction digestions would indicate the occurrences of homology-directed repair and the likelihood for the cells to harbour the E381K mutation. Purified PCR amplicons from the DNA of clones that harboured the silent mutation at PAM2 were analysed by DNA sequencing.

2.4 Protein analysis methods

2.4.1 Protein extraction from cell cultures

The cells cultured in 6-well plates were washed with PBS and lysed with 300µl of protein extraction buffer (10mM Tris, 100mM NaCl, 1mM EDTA, 1% Triton X-100, pH7.4) supplemented with protease inhibitor cocktail at 1 in 100 dilution (Sigma). The cell lysates were incubated at 4°C for 1 hour and were then centrifuged at 15000rpm for 15 minutes to remove debris. Subsequently, the supernatants containing the extracted proteins were kept and the protein concentrations were determined by the Pierce™ BCA protein assay kit (Fisher Scientific, 23225).

2.4.2 Western blotting analysis

Equal amounts of protein (10-50µg of total protein) were denatured in an appropriate preparation containing LDS sample buffer (Invitrogen) and NuPAGE reducing agent (Invitrogen) at 90°C for 10 minutes. The samples and SeeBlue® Plus2 Pre-Stained Standard (Invitrogen) were loaded into precast NuPAGE 4-12% Bis-Tris gels and resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using the XCell SureLock™ Mini-Cell Electrophoresis System and associated reagents from Invitrogen. The gels were run in MOPS SDS running buffer at 100V, and the proteins were electro-transferred on to nitrocellulose membranes in NuPAGE transfer buffer at 30V for 2 hours.

Non-specific binding of proteins was blocked with 5% marvel (milk powder in PBS-0.1% Tween 20, PBST) for 1 hour at room temperature. The membranes were incubated with primary antibodies diluted in 2% marvel at 4°C overnight, and then

washed with PBST for 30 minutes. Subsequently, the membranes were incubated with appropriate secondary horseradish peroxidase (HRP)-conjugated antibodies diluted in 2% marvel at room temperature for 1 hour, and then washed 3 times for 30 minutes again. If necessary, hydrogen peroxide was used to inactive the peroxidase activities to allow for sequential re-probing of western blots (Sennepin et al., 2009). Table 2-23 provides a summary of the antibodies used.

Table 2-23 Antibodies used in western blotting

Primary antibodies			
Antigen	Host/Clone	Dilution	Source
α -tubulin	Mouse monoclonal	1 in 5000	Sigma T5168
AChR δ -subunit	Goat polyclonal	1 in 500	Santa Cruz sc-1451
GFP	Rabbit polyclonal	1in 2000	Abcam ab6556
mCherry	Rabbit polyclonal	1 in 2000	Abcam ab167453
Rapsyn	Mouse monoclonal	1 in 200	Abcam ab11423
Rapsyn	Rabbit polyclonal	1 in 500	Abcam ab58662
HRP-conjugated secondary antibodies			
Antigen	Host/ Clone	Dilution	Source
Goat IgG	Rabbit polyclonal	1 in 2500	Dako P0449
Mouse IgG	Goat polyclonal	1 in 2500	Dako P0447
Rabbit IgG	Goat polyclonal	1 in 2500	Dako P0448

ECL or ECL Prime reagents (VWR) were used to develop the bound HRP-conjugated antibodies, and protein bands were detected by exposing the membranes to autoradiography films (Fisher Scientific). Images of the developed films were taken with the GelDoc-It2 310 imaging system and its associated VisionWorks®LS image acquisition and analysis software (UVP). The relative intensities of the protein bands were quantified by densitometry analysis using Fiji.

2.4.3 Cycloheximide chase assay

To determine the stability of rapsyn-EGFP variants, HEK 293 cells plated on 6-well plates were transfected with pEGFP-N1-*RAPSN* constructs and pcDNA3.1-mCherry as a control for transfection efficiency. Thirty-six hours after the transfections, cycloheximide (Sigma, C4859) was added to the culture medium of the cells at the final concentration of 20µg per ml. The cells were lysed at 0, 2, 4, 8, 16 and 24 hours after exposure to cycloheximide.

2.4.4 Agrin-induced AChR clustering

Concentrations of agrin that induced the maximum amount of AChR clustering were determined by an agrin titration experiment on *Rapsn*-KO myotubes expressing WT-rapsyn-EGFP or C2C12 myotubes. The typical range of agrin used in experiments was between 1 in 100 to 1 in 10 dilutions from the original stock.

Myotubes cultured in 6-well plates were incubated with agrin in culture medium for 16 hours to induce AChR clustering. To induce the disassembly of AChR clusters, carbamoylcholine chloride (carbachol, Sigma, C4382) was added to the differentiation medium of myotubes after agrin stimulation. The AChR clusters formed were labelled with fluorescently conjugated α -bungarotoxin (α -BuTx) diluted in the medium for 1 hour under culture conditions. Subsequently, excess α -BuTx was removed by 3x 5-minute washes with the culture medium, and the labelled myotubes were fixed in 3% paraformaldehyde (Sigma) in the culture medium for 15 minutes at room temperature. The myotubes were washed 3 times with PBS, and AChR clusters were assessed by fluorescence microscopy. Table 2-24 provides a summary of the fluorescently conjugated α -BuTx used to label AChR.

Table 2-24 Fluorescently-conjugated α -bungarotoxin used to label AChR on cultured myotubes

Fluorescently conjugated α -bungarotoxin			
Target	Alexa Fluor® conjugate	Dilution	Source
AChR	α -BuTx-488	1 μ g/ml	Invitrogen B-13422
AChR	α -BuTx-594	1 μ g/ml	Invitrogen B-13423

To detect for rapsyn-HaloTag or rapsyn-SNAPTag clusters, the myotubes were labelled by incubation with HaloTag-TMR (Promega, G8252) or SNAP505* (NEB, S9103) respectively. Both substrates are cell permeable and were applied to live cells at 5 μ M in the culture medium, for 15 minutes for HaloTagTMR and 30 minutes for SNAP505*, under culture conditions. Excess substrates were washed off by 3 media changes and AChR clusters were labelled with fluorescently conjugated α -BuTx, after which the cells were fixed with 3% paraformaldehyde for subsequent analyses.

2.4.5 Cell-surface 125 I- α -bungarotoxin binding assay

The levels of AChR on the surfaces of cells cultured in 6-well plates were measured by the 125 I- α -BuTx binding assay. For transfected HEK 293 cells, the assay was performed on cells 48 hours post-transfection. To ensure the reproducibility of results, each assay analysed triplicate wells of cells for each condition. In experiments that estimated the background levels of radioactivity, the cells were incubated with an excess amount (5 μ g/ml) of unlabelled α -BuTx (Invitrogen, B1601) diluted in blocking solution (10% bovine serum albumin, BSA, in PBS) for 15 minutes before 125 I- α -BuTx (PerkinElmer, NEX126050UC) was applied.

In the assay, each well of cells was washed 3 times with PBS, followed by incubations with 500µl of ^{125}I - α -BuTx at 10^6 cpm/ml diluted in blocking solution for 1 hour at room temperature with gentle rocking. Subsequently, the cells were washed 3 times for 5 minutes each with 1ml of PBS and then dissolved in 500µl of protein extraction buffer. The cell extracts were transferred to an eppendorf tube and the amounts of ^{125}I - α -BuTx bound were quantified by a gamma-counter.

2.4.6 Immunofluorescence on teased muscle fibres

The morphology of endplate AChR clusters on the diaphragm, extensor digitorum longus (EDL), soleus and tibialis anterior (TA) muscles of $\text{hy}^+ \epsilon^{\text{het}}$ control and $\text{hy}^+ \epsilon^{-/-}$ mice, dissected by Dr. Richard Webster, were analysed. To label AChR clusters, intact muscles were incubated with tetramethylrhodamine (TMR)-conjugated α -BuTx (α -BuTx-TMR) diluted in PBS at room temperature for 30 minutes. To detect both acetylcholinesterase and AChR, muscle fibres were incubated with Alexa Fluor® 488-conjugated fasciculin 2 (fasciculin 2-488) and α -BuTx-594 in PBS for 30 minutes. Subsequently, the muscles were washed for 6x 10 minutes with PBS to remove excess toxins and fixed in 1% paraformaldehyde in PBS for 30 minutes, followed by further washing with PBS at 4°C overnight.

To reveal the neuromuscular junctions, the EDL, soleus and TA muscles were initially divided into bundles. Subsequently the muscle fibres were teased and mounted onto superfrost slides (VWR) in confocal-UV-matrix (Micro tech lab). Since diaphragm muscles are relatively thin, they were mounted directly without the teasing of muscle fibres. The dilutions and sources of fluorescently conjugated neurotoxins are summarised in Table 2-25.

Table 2-25 Antibodies and neurotoxins used to label endplate proteins

Fluorescently conjugated neurotoxins			
Target	Fluorescent conjugate	Dilution	Source
AChR	α -BuTx-TMR	5 μ g/ml	Invitrogen T-1175
Acetylcholinesterase	Fasciculin 2-488	2 μ g/ml	Invitrogen (special order)
Primary antibodies			
Antigen	Host/Clone	Dilution	Source
Neurofilament	Chicken polyclonal	1 in 200	Abcam ab4680
Rapsyn	Rabbit polyclonal	1 in 100	Abcam ab58662
Alexa Fluor®488-conjugated secondary antibodies			
Antigen	Host/Clone	Dilution	Source
Chicken IgG	Donkey polyclonal	1 in 200	Jackson ImmunoResearch Inc. 703-545-155
Rabbit IgG	Goat polyclonal	1 in 200	Invitrogen A-11029

For antibody labelling in muscle fibres, intact diaphragm muscles and teased bundles of EDL, soleus and TA muscles labelled with α -BuTx-TMR and fixed were dehydrated with absolute ethanol, followed by methanol at -20°C for 10 minutes each. The muscle bundles were permeabilised with 0.1% Triton X-100 in blocker solution (3% normal goat serum in PBST) at room temperature for 15 minutes and then washed for 2 hours with at least 3 changes of PBST. Subsequently, the primary antibodies diluted in blocker solution were applied to the muscles overnight at 4°C. On the following day, excess primary antibodies were removed by washing with 10 changes of PBST within 2 hours, and the muscles were incubated with appropriate fluorescently conjugated secondary antibodies diluted in blocker solution for 3 hours at room temperature. The dehydration protocol disrupted the fluorescence of Fasciculin 2-488 when it was applied to muscles before fixing. Therefore it should be applied together with secondary antibodies if the labelling of acetylcholinesterase is desired. After washing overnight in PBST at 4°C, the muscles were mounted.

2.5 *In vivo* methods

To evaluate the efficacy and mechanisms of salbutamol therapy, a mouse model of AChR deficiency syndrome ($h\gamma^+\epsilon^{-/-}$ mice) was used (Cossins et al., 2004). These transgenic C57BL/6 mice lack the expression of AChR ϵ -subunit, but were rescued from the lethality of the gene knockout by the expression of human AChR γ -subunit under a human skeletal α -actin promoter. Dr. Richard Webster was responsible for breeding the $h\gamma^+\epsilon^{-/-}$ mice used in this study.

The genotypes of the mice were confirmed by PCR analysis of genomic DNA extracted from ear-clips of the mice using DirectPCR lysis reagents (ViagenBiotech, 401-E) supplemented with proteinase K (bioWorld, 21560024). This was mostly performed by Miss Susan Maxwell and occasionally by me. In addition to *CHRNA* and *Chrne*, PCR to detect the neomycin resistance gene (*neo*) was performed to evaluate the homozygosity of the disruption to *Chrne*. The desired genotype of $h\gamma^+\epsilon^{-/-}$ mice from PCR analysis, using primers listed in Table 2-26, should be *Chrne*^{-/-}, *neo*^{+/+} with detectable *CHRNA* from the cDNA of human AChR γ -subunit.

Table 2-26 Sequences of oligonucleotides used for genotyping $h\gamma^+\epsilon^{-/-}$ mice

Target gene	Primer name	Primer sequence (5' to 3')
<i>CHRNA</i>	CHRNA F	GAGTTAGGGCTGAGCCAGTTCTGTG
	CHRNA R	GTCTGGTGAGGGCAGGTAGGGGCGTG
<i>Chrne</i>	Chrne F	CTCTTCGACAATTATGATCCAGA
	Chrne R	GAGCCGATAGTCGTGCCAGT
<i>neo</i>	neo F	CAACAGACAATCGGCTGCTCT
	neo R	GAATGGGCAGGTAGCCGGAT

The design of the treatment trial and regimens tested are described in Chapter 6.2. Briefly, the mice were subjected to treatment for 10 weeks, and their weight and muscle strength were measured once a week. Pyridostigmine bromide (Sigma, 29797) and/or salbutamol sulphate (Sigma, 59803) were dissolved in drinking water, which was available to the mice 24 hours per day. The medicated drinking water was refreshed once a week. At the end of the treatment period, the diaphragm, extensor digitorum longus (EDL), soleus and tibialis anterior (TA) muscles were dissected, and the endplates were labelled with fluorescent α -BuTx conjugates and analysed.

2.5.1 Evaluation of muscle strength of mice

The muscle strength and fatigability of $hy^+ \epsilon^{-/-}$ mice were evaluated once a week by the inverted screen test (Cossins et al., 2004). In the test, a mouse was placed on a 40cm² screen that consists of wire mesh and the screen was inverted so the mouse was hanging upside down above a padded surface. The duration that the mouse was able to hang on to the screen in 3 continuous attempts was recorded.



Figure 2-5 The inverted screen test used to measure muscle strength

The set up for the inverted screen test is shown, and to evaluate strength and fatigability, the performances from 3 continuous attempts without rest were recorded.

2.6 Microscopy analysis

2.6.1 Quantifications of AChR clusters formed on myotubes

Images used for the quantification of AChR clusters labelled with α -BuTx-594 on cultured myotubes were acquired at 20x magnification using an Olympus IX71 fluorescence microscope with SimplePCI software (Digital Pixel).

Agrin-induced AChR clusters were initially quantified for the number of AChR clusters per field and the lengths of the clusters that were longer than $3\mu\text{m}$ using Volocity (Improvision). The analysis was replaced by Fiji macro analysis.

The design of the macro for the analysis of AChR clusters (Figure 2-6) is described in Chapter 3-2. In this method, the number, lengths, average area and total area of AChR clusters, larger than $2.5\mu\text{m}^2$, per field were quantified.

```
1 title = getTitle();
2 run("Colors...", "foreground=black background=white selection=yellow");
3 roiManager("reset");
4 run("Set Scale...", "distance=312 known=100 pixel=1 unit=um");
5 run("8-bit");
6 run("Duplicate...", "title=image-2");
7 run("Duplicate...", "title=image-1");
8 run("Gaussian Blur...", "sigma=x");
9 imageCalculator("Subtract create", "image-2", "image-1");
10 setThreshold(x, 255);
11 run("Convert to Mask");
12 run("Analyze Particles...", "size=2.5-Infinity circularity=0.00-1.00 show=[Overlay Outlines] display summarize add");
13 roiManager("Save", "/Users/"+title+"-RoiSet.zip");
14 run("Flatten");
15 selectWindow("image-1");
16 close();
17 selectWindow("image-2");
18 close();
```

Figure 2-6 Macro for the analysis of AChR clusters on cultured myotubes

The analysis was performed in Fiji with the macro. The Gaussian blur and threshold values marked by x in lines 8 and 10 respectively were optimised for specific experiments.

2.6.2 Fluorescence recovery after photobleaching (FRAP)

To determine the mobility of rapsyn and AChR labelled with α -BuTx-594 within clusters, FRAP data were acquired using a Zeiss 780 inverted microscope with Zeiss efficient navigation (ZEN) 2011 software.

The protocol and analysis is described in Chapter 4.5.1. To supplement, images (12bits/pixel) were acquired using a 100x objective (Plan-Apochromat 100x/1.40 Oil M27) at scan speed 9 and 256 x 256 pixel frames with the resolution of 100nm/pixel. 25 pre-bleach images were acquired to evaluate the steady state fluorescence intensity of the clusters. Photobleaching of rapsyn-EGFP and α -BuTx-594 at a predefined region (4 μ m x 1 μ m were fixed at the centre of the image frame) was performed by 488nm and 594nm lasers respectively at 100% power and 20 continuous iterations. Subsequently, 175 frames were acquired to monitor changes in the fluorescence intensities. The intensities of regions (4 μ m x 2 μ m) that separately cover the bleached area and a no-bleach control region were measured by Fiji (Figure 2-7).

```
1 im = getTitle()
2 setSlice(1);
3 run("Specify...", "width=40 height=20 x=111 y=98 slice=1");
4 run("Plot Z-axis Profile");
5 Plot.getValues(xvalues,yvalues);
6 yvalue =im+"_grn \t";
7 for(i=0;i<xvalues.length;i++){
8 yvalue = yvalue+yvalues[i]+" \t";
9 }
10 File.append(yvalue, getDirectory("home")+"/Desktop/profileGrn1.xls");
11 selectWindow(im);
12
13 setSlice(2);
14 run("Specify...", "width=40 height=20 x=111 y=98 slice=2");
15 run("Plot Z-axis Profile");
16 Plot.getValues(xvalues,yvalues);
17 yvalue =im+"_red \t";
18 for(i=0;i<xvalues.length;i++){
19 yvalue = yvalue+yvalues[i]+" \t";
20 }
21 File.append(yvalue, getDirectory("home")+"/Desktop/profileRed.xls");
```

Figure 2-7 Macro for FRAP analysis of rapsyn-EGFP and AChR within clusters

The analysis was performed in Fiji using the macro. The intensities measured from image frames 1-5, 26-30 and 195-200 of the 200 frames of each cluster were averaged to represent the maximum, minimum and recovery fluorescent intensities.

2.6.3 Analysis of the morphology of endplates

Endplate AChR, labelled with α -BuTx-594, was imaged at 40x magnification using an Olympus IX71 fluorescence microscope with the SimplePCI software.

The design of the analysis of the morphology of endplates is described in Chapter 6.1.1. Briefly, the total area of α -BuTx-594 bound regions, the number and average size of α -BuTx-bound fragments per endplate were analysed by Fiji. The measurements in pixels were converted to μm^2 according to the imaging settings of '6.24pixels/ μm ' at 40x magnification. The macro used is shown in Figure 2-8.

```
1 roiManager("reset");
2 run("Add to Manager");
3 title = getTitle();
4 path = getDirectory("image");
5 run("Duplicate...", "title=image-2");
6 run("Set Scale...", "distance=0 known=0 pixel=1 unit=pixel");
7 run("8-bit");
8 run("Duplicate...", "title=image-1");
9
10 run("Gaussian Blur...", "sigma=20");
11 imageCalculator("Subtract create", "image-2", "image-1");
12 selectWindow("Result of image-2");
13 setThreshold(5, 255);
14 run("Convert to Mask");
15 run("Duplicate...", "Result of image-2");
16 selectWindow("Result of image-1");
17 rename("area");
18 run("Analyze Particles...", "size=5-Infinity circularity=0.00-1.00 show=Outlines display summarize");
19
20 selectWindow(title);
21 roiManager("Select",0);
22 close();
```

Figure 2-8 Macro for the analysis of the morphology of endplate AChR clusters

The analysis of manually selected α -BuTx-bound regions (endplates) within an image was performed in Fiji. Subsequently, the results in pixels were converted to μm^2 .

2.6.4 Stimulated emission depletion microscopy (STED)

Confocal images (12bit/pixel) were acquired at 100nm²/pixel and line averaged 4 times using a Leica SP8 Gated STED microscope with a 100x objective (HC PL APO CS2 100x/1.40) and the Leica Application Suite-Advanced Fluorescence (LAS AF) software.

Adapting the set-up described for confocal microscopy, STED images were acquired at system optimised resolution by gated-STED with the time gating set at 1.5nsec to eliminate signals emitted before the STED lasers take effect. AChR clusters on myotubes were labelled with α -BuTx-TMR and imaged using the STED660 laser. 2 colour STED images of endplates were acquired at system-optimised resolution and sequentially with the STED660 laser for AChR labeled with α -BuTx-TMR, and then the STED592 laser for the acetylcholinesterase labelled with Fascifulin2-488.

2.7 Statistical analyses

Statistical calculations were performed with GraphPad Prism 6. The specific statistical tests used are described in the figure legends of the respective experiments. In all cases, $p < 0.05$ was used as a predetermined value for statistical significance.

Chapter 3

Pathogenicity of *RAPSN* mutations in congenital myasthenic syndromes

3.1 Introduction

The clinical spectrum of *RAPSN*-CMS results from reduced AChR clustering that causes AChR deficiency at the postsynaptic NMJ. While screening for the common mutation N88K facilitates the genetic diagnoses made for the majority of *RAPSN*-CMS patients, it can be more difficult in cases where patients do not harbour N88K. The lack of genotype-phenotype correlation for *RAPSN* mutations identified and life-threatening episodic crises are also unresolved issues. Therefore, increased knowledge and documentation on the pathogenic properties of *RAPSN* mutations would positively impact disease diagnosis and genetic counselling.

The aims of this chapter are to confirm the pathogenicity of *RAPSN* mutations by an assay based on AChR clustering, and to identify clues towards correlation between the results in AChR clustering *in vitro* and clinical phenotypes. The objectives are as follows:

- 1) Develop an efficient and consistent method of analysis for AChR clustering on cultured myotubes
- 2) Determine the effects of *RAPSN* mutations on AChR clustering

3.2 Establishing parameters for the analysis of rapsyn function based on AChR clustering

The artificial expression of AChR subunits and rapsyn-variants in heterologous cells to study the expression of rapsyn and AChR clusters on cell surfaces has provided mechanistic insights into the AChR clustering defects caused by *RAPSN* mutations (Cossins et al., 2006; Muller et al., 2006a; Ohno et al., 2002). However, it has been stated that such expression studies do not always consistently reflect AChR expression at the motor endplate (Beeson et al., 2005). Agrin-induced AChR clustering in cultured myotubes is a widely used model for investigating AChR clustering as it mimics the events that can occur at the postsynaptic membrane (Finn et al., 2003; Weston et al., 2000; Yang et al., 2011). The overexpression of *RAPSN*-variants in a *Rapsn*-knockout (KO) muscle cell line allows the AChR clustering properties of specific rapsyn variants to be studied in the absence of endogenous rapsyn (Cossins et al., 2006). Therefore, the previously established model was used to assess rapsyn-AChR clustering functions in this thesis.

The expression of rapsyn variants in *Rapsn*-KO cells is achieved by using the pBabe-PURO retroviral system. cDNA of rapsyn variants were cloned into the retroviral expression vector pBabe-PURO-EGFP so that rapsyn tagged with enhanced green fluorescent protein (EGFP) at its C-terminus is expressed for easy identification. Retroviruses used to introduce rapsyn-EGFP into *Rapsn*-KO cells are produced by transfection of plasmids into the retroviral packaging cell line, GP +E86 cells; and stable expression of rapsyn-variants can be achieved by puromycin selection after retroviral infection. The myoblasts can then be seeded for differentiation into myotubes and subsequently stimulated with agrin to induce AChR clustering. AChR

clusters on the surface of myotubes are identified by α -bungarotoxin (BuTx)-594 (Figure 3-1 A summarises the key stages of the assay), where the colocalisation between rapsyn-EGFP and α -BuTx provides further confirmation for the identities of AChR clusters (Figure 3-1 B).

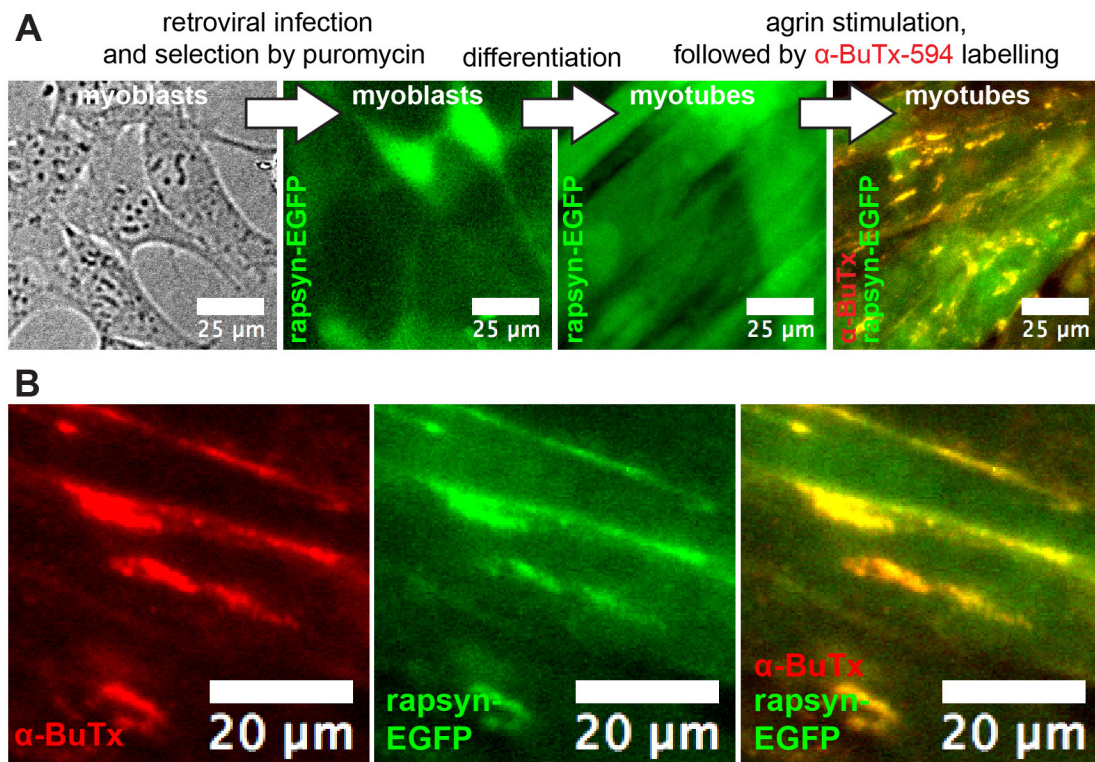


Figure 3-1 Summary of the methodology of an assay based on AChR clustering

- (A)** Representational images on the various stages of the *Rapsn*-KO cells in the AChR clustering assay, from the generation of myoblast cultures that stably express rapsyn-EGFP to the induction of agrin-induced AChR clustering on myotubes
- (B)** Colocalisation between AChR labelled with α -BuTx-594 (red) and rapsyn-EGFP (green) confirms AChR clusters

3.2.1 The Fiji macro analysis for AChR clustering

Quantification of AChR clusters has previously been performed using Volocity® 3D analysis software (Volocity analysis). The analysis requires measurement lines to be manually drawn over each of the AChR clusters present in the microscopic field Figure 3 A-B. The total number of measurement lines drawn on an image represents the number of clusters per field and the lengths of the measurement lines will indicate the size of the AChR clusters. The morphology of AChR clusters on myotubes can be heterogeneous and variable in size and shape. Even though there is a standardised cut off in our laboratory for AChR clusters to be longer than 3µm, judgments made about what a cluster is on an image can vary between observers. A microscopic field at 20x magnification corresponds to 430.77µm x 328.21µm, can yield over 200 AChR clusters on *Rapsn*-KO myotubes that express wild-type (WT) rapsyn-EGFP. Therefore, the quantification of AChR clusters is time-consuming process.

As the protocol for Volocity analysis is limited by the subjectivity of the human observer, I have explored the possibility of an alternative approach to quantify AChR clusters. With generous advice from Dr. Dominic Waithe, the image analyst of the Wolfson Imaging Centre Oxford, I have developed a computerised method to objectively quantify AChR clusters by Fiji macro analysis. Fiji is open-source software that allows for biological-image analysis (Schindelin et al., 2012). The macro designed contains a series of commands that aim to remove the background and isolate individual AChR clusters on images for quantification. Once the parameters for specific experiments have been established, the use of the macro allows automated analysis of batches of images, making it a time efficient process.

The analyses of individual images can be saved and reviewed after the analysis to confirm the accuracy of the results.

There are three key steps in the macro that act to guide the computer software in identifying AChR clusters (Figure 3-2). 1) Background subtraction: labelled AChR clusters have higher fluorescence intensity than the background, therefore an AChR cluster can be defined by its high-frequency details on an image plane. The application of a Gaussian blur filter to an image acts to define its background fluorescence, as the filter removes information of high-frequency details, i.e. AChR clusters. To remove the background, information on the filtered image is subtracted from the original. 2) Threshold adjustment: this step removes the variable intensities across the field and results in the isolation of AChR clusters on a binary image. 3) Quantification of AChR clusters: the number and size of clusters are analysed and clusters that are smaller than $2.5\mu\text{m}^2$ are excluded. The accuracy of the macro in identifying AChR clusters depends on the combination of values used for the Gaussian blur filter and the threshold adjustments. These values are determined by trial and error on numerous images until a combination that makes sensible estimations on AChR cluster and size is found. Once determined, the same set of values will be used to analyse all images for the same experiment to reduce bias and inconsistency in the results.

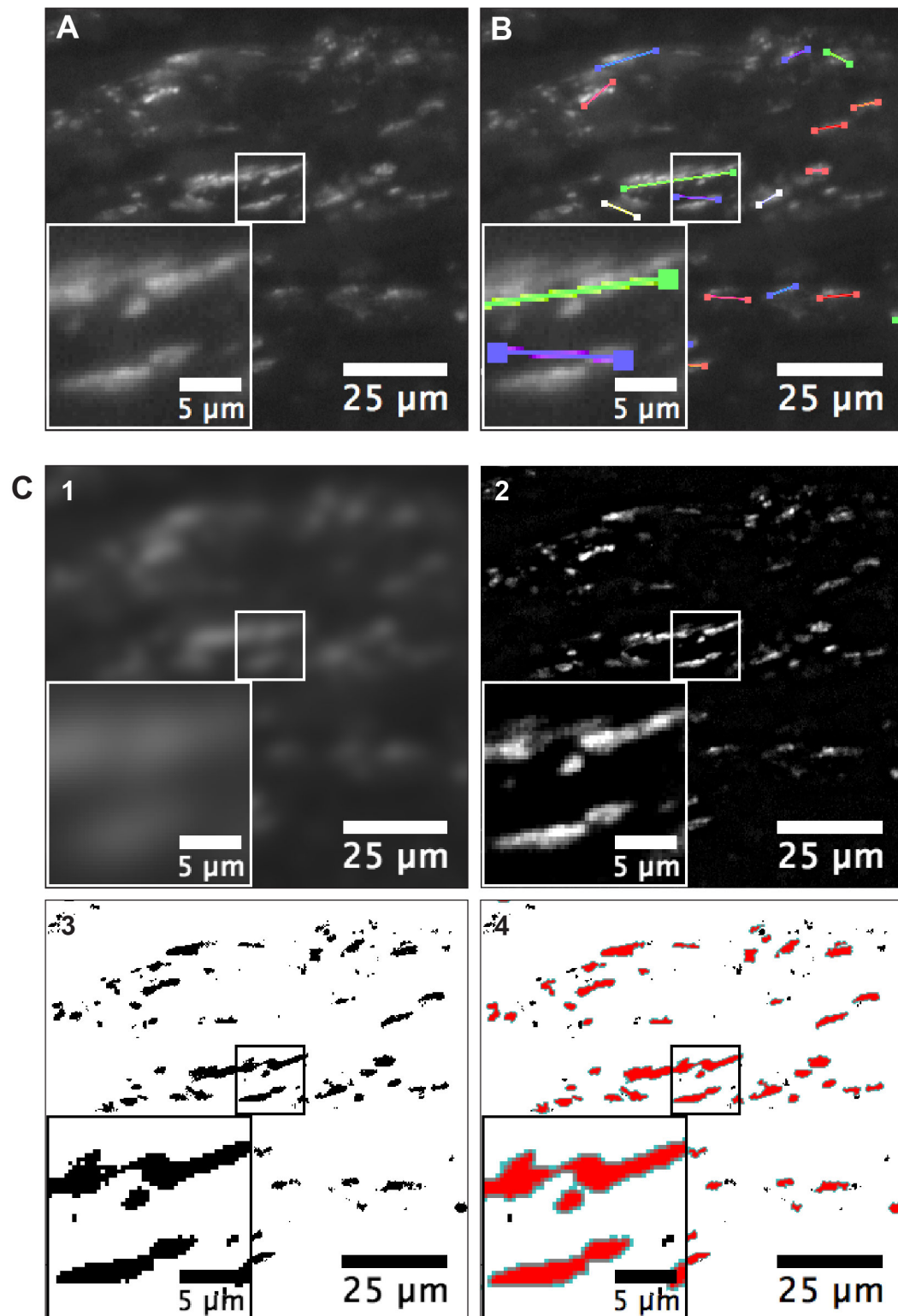


Figure 3-2 Methods of quantifying of AChR clusters labelled with α -BuTx-594

(A) Part of a microscopic field subjected to image analysis (original image)

(B) Volocity analysis by measurement lines

(C) Fiji macro analysis: (1) Gaussian blur filtered image (2) Image after background subtraction (3) Thresholded binary image (4) AChR clusters quantified in red

3.2.2 Detection of AChR clusters by Fiji macro analysis

An agrin titration experiment on C2C12 myotubes was performed to evaluate the ability of Fiji macro analysis to accurately identify AChR clusters. C2C12 is a muscle cell line that expresses endogenous rapsyn, making it a convenient option to generate AChR clusters for quantifications. A gradient of five dilutions from the original stock of full-length human agrin (FLagrin) were applied to the myotubes, as agrin-induced AChR clustering is known to occur in a concentration dependent manner. Subsequently, the AChR clusters formed were labelled with α -BuTx-594, and 10 images for each condition were taken and analysed by the macro (Figures 3-3 and 3-4).

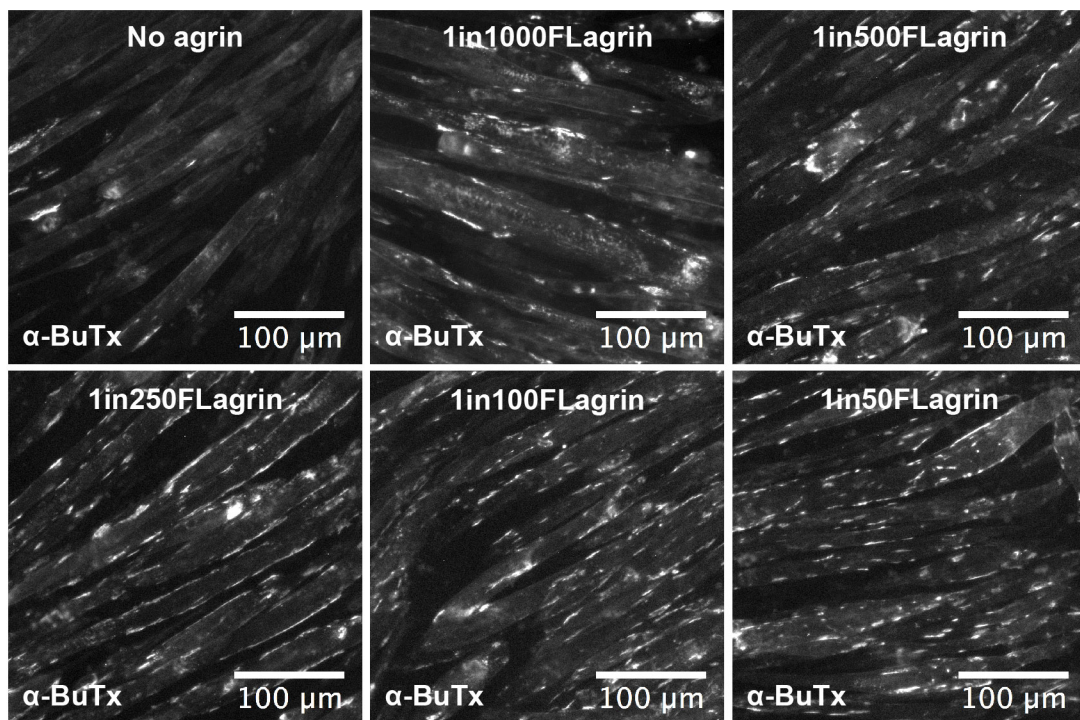


Figure 3-3 Example images of AChR clusters in an agrin-titration experiment

Different concentrations of human full-length agrin (FLagrin): 1 in 1000, 1 in 500, 1 in 250, 1 in 100 and 1 in 50, and a no agrin control, were applied to C2C12 myotubes for 16h. The cells were then incubated with α -BuTx-594 to label AChR clusters.

The images shown represent part of a microscopic field at 20x magnification.

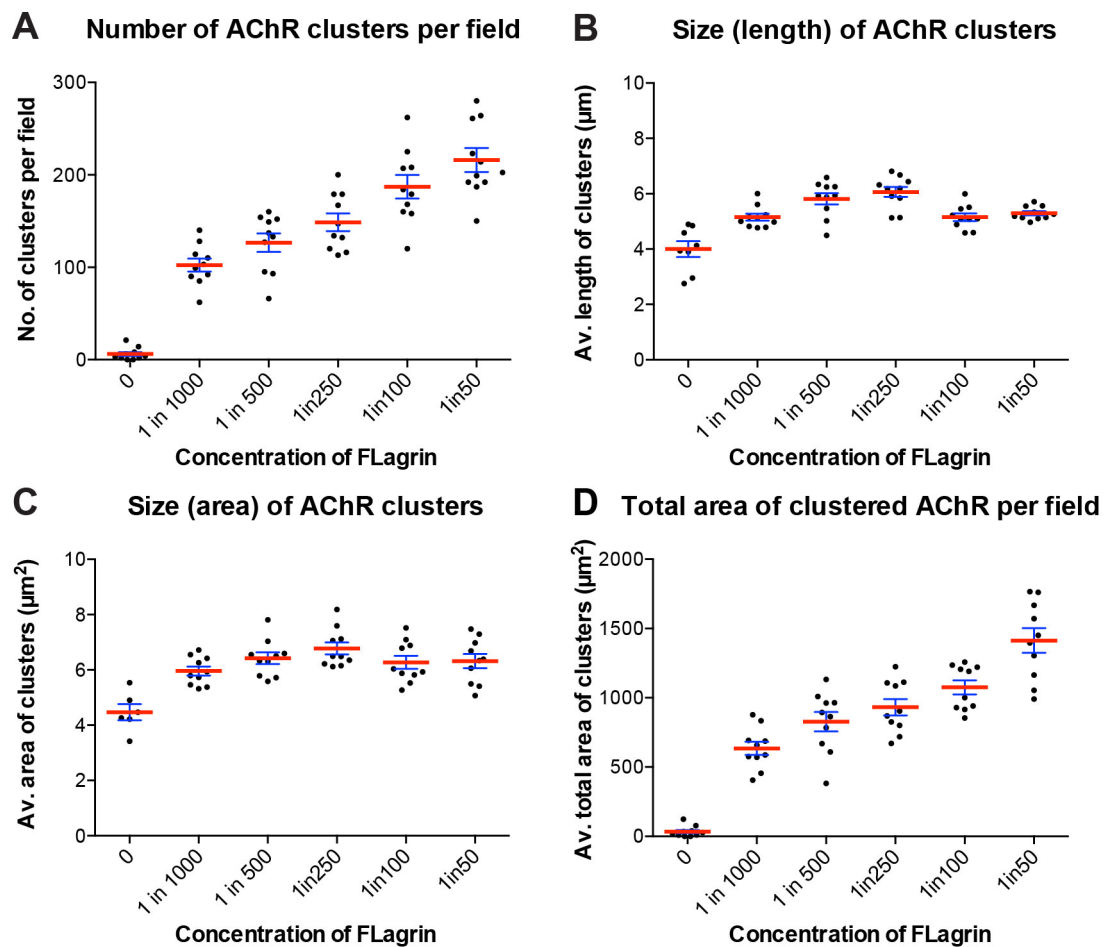


Figure 3-4 Fiji macro analysis of the effects of agrin on AChR clustering

C2C12 myotubes were treated with a gradient of concentration of FLaGrin to stimulate AChR clustering, and AChR clusters were labelled with α -BuTx-594. 10 images per condition were analysed by Fiji macro analysis. The data points represent data from single images and are summarised by mean $\pm$ SEM for each condition.

Figures 3-3 and 3-4 show that the results obtained by Fiji macro analysis are as expected, with a few AChR clusters formed on myotubes without FLaGrin, and more AChR clusters formed when stimulated by FLaGrin at higher concentrations. Differences in size (length and area) of AChR clusters were often subtle and less easily identified. The total area occupied by AChR clusters per microscopic field resembles the data on the number of clusters per field, as it provides a summary on the data from the number and size of AChR present per microscopic field.

3.2.3 Specificity of Fiji macro analysis in detecting AChR clusters

The previous experiment has confirmed that Fiji macro analysis offers sufficient sensitivity to identify the differences between images of AChR clusters. The next step is to confirm its specificity in identifying clusters, as α -BuTx-594 can bind to cell debris, which yields fluorescence signals that can be misinterpreted as AChR clusters.

The *Rapsn*-KO cells (untreated) were used because they do not form AChR clusters, and therefore they are able to generate images that are expected not to contain AChR clusters. In addition, *Rapsn*-KO cells introduced to express EGFP and WT-rapsyn-EGFP were also included in the experiment. FLa-grin was applied to untreated, EGFP and WT-rapsyn-EGFP myotubes to stimulate the AChR clustering process. A no agrin control to WT-rapsyn-EGFP was also performed to illustrate that the AChR clustering process in these cells is also agrin dependent.

Western blotting analyses from whole cell lysates of myotubes confirmed the expression of rapsyn-EGFP in cells that were infected with WT-rapsyn-EGFP; and that there is no detectable rapsyn-EGFP present in untreated *Rapsn*-KO cells or cells that were infected with EGFP. A total of 60 images from 3 experiments were analysed by both Volocity and Fiji macro analyses to compare results. The Volocity analysis was performed by blinded assessment to avoid bias (Figures 3-5 and 3-6).

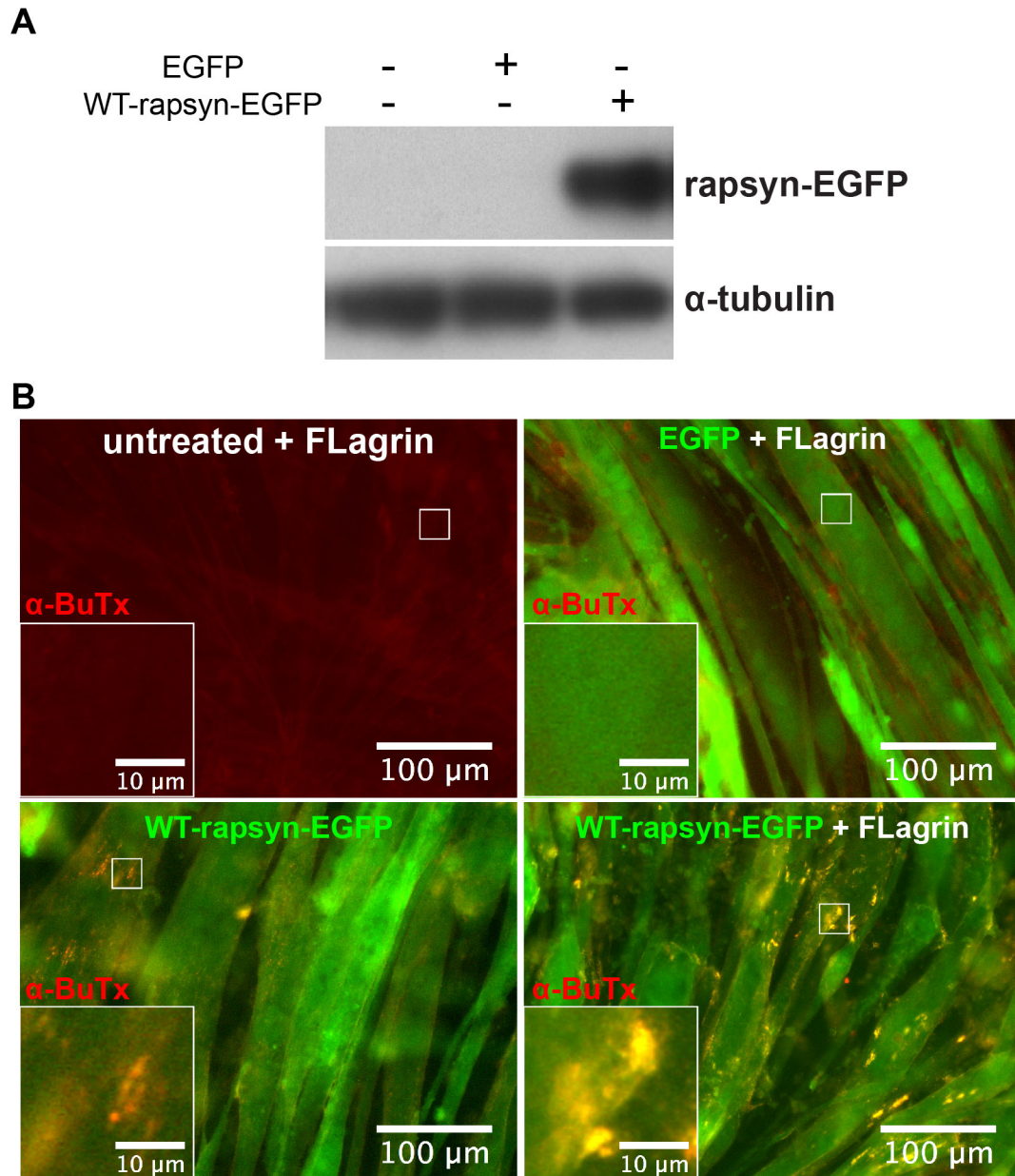


Figure 3-5 The effect of WT-rapsyn-EGFP expression on AChR clustering

Rapsn-KO cells were infected with retroviruses to induce the expression of EGFP or WT-rapsyn-EGFP. The cells were differentiated into myotubes and incubated with FLagrin to induce AChR clustering. AChR were labeled with α -BuTx-594.

(A) Cell lysates from the myotubes of *Rapsn*-KO cells, untreated or infected to express EGFP or WT-rapsyn-EGFP, were analysed by western blotting to confirm the expression of rapsyn-EGFP.

(B) Untreated and EGFP-expressing myotubes did not form AChR clusters when they were treated with FLagrin. The expression of WT-rapsyn-EGFP in *Rapsn*-KO cells induces a small number of spontaneous clusters and more AChR clusters were formed by FLagrin stimulation.

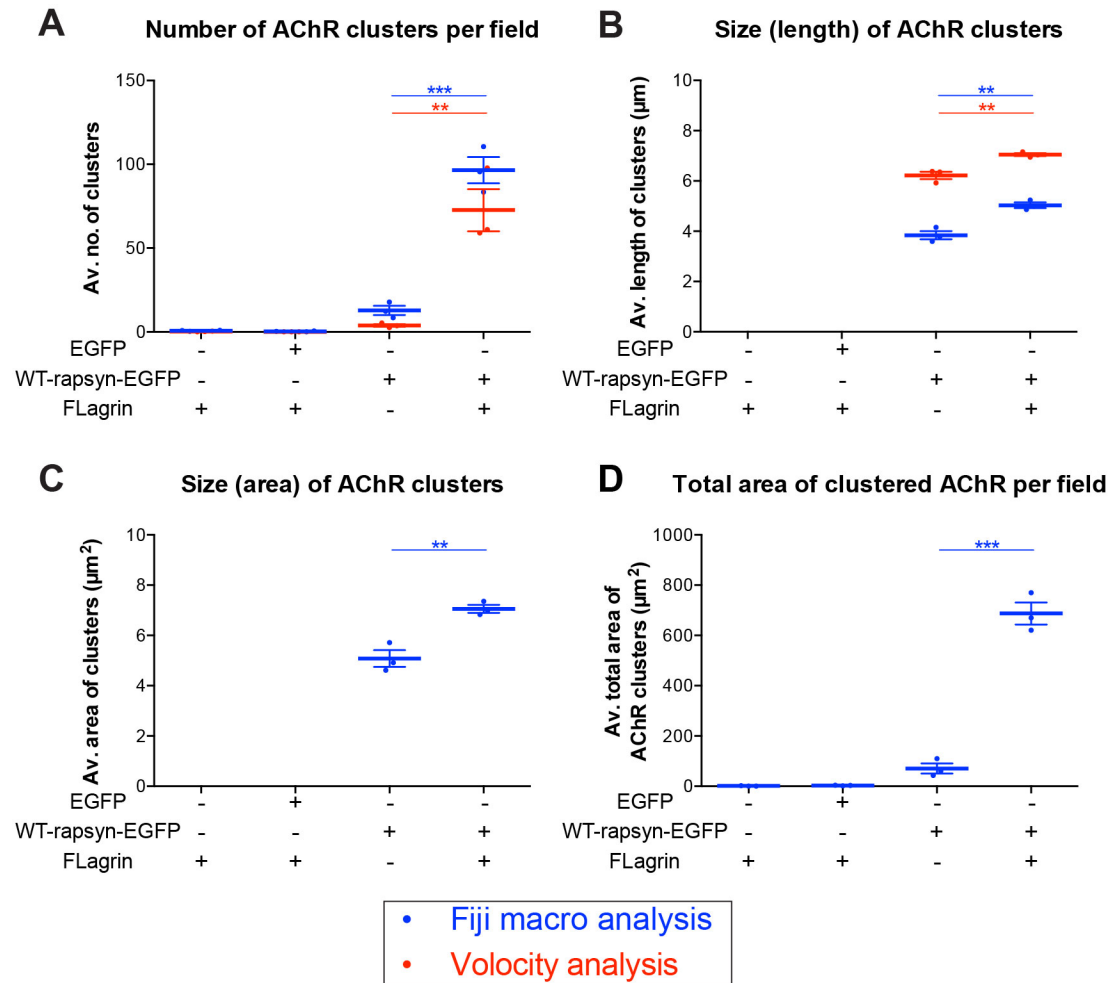


Figure 3-6 Quantification of AChR clusters in the *Rapsn*-KO model system

Rapsn-KO cells were infected with EGFP or WT-rapsyn-EGFP. Differentiated myotubes were treated with FLaigrin to stimulate AChR clustering, and AChR were labeled with α -BuTx-594. For each condition, 60 images from 3 experiments were analysed by both Volocity and Fiji macro analysis.

The data points represent the mean of the data from 20 images for each condition, and the results are summarised by overall mean $\pm$ SEM. Asterisks *** indicate $p < 0.001$; ** for $p < 0.01$; statistical analyses were performed by the two-tailed unpaired t test.

On *Rapsn*-KO myotubes and cells that were infected with EGFP only, both Volocity and Fiji macro analysis detected an average of less than 1 AChR cluster per microscopic field. These were considered to be non-specific binding of α -BuTx. On myotubes expressing WT-rapsyn-EGFP, both methods detected significantly more AChR clusters when the cells were incubated with FLaGrin than the number of spontaneous AChR clusters formed.

FLagrin-induced AChR clusters were significantly longer than spontaneous clusters. Area measurements were not offered by Volocity analysis, and Fiji macro analysis identified both the average area of individual clusters and the total area of FLaGrin-induced AChR clusters per field to be significantly larger than spontaneous clusters.

Although both methods of analysis were able to detect the same differences in the properties of AChR clusters formed under the different conditions, Fiji macro analysis reported a higher number of agrin-induced AChR clusters on myotubes expressing WT-rapsyn-EGFP ($p=0.0239$). This variation is acceptable, as there can also be variation in the results analysed by different human observers. On the other hand, Volocity analysis suggested that the lengths of both spontaneous and FLaGrin-induced AChR clusters were longer than what was reported by Fiji macro analysis ($p<0.0001$). These differences are likely to stem from the technical difficulty in Volocity analysis to precisely place the anchor points of the measurement lines at each end of individual AChR clusters. Regardless of this, the patterns of results generated by both methods are consistent, suggesting Fiji macro analysis could be used as an alternative method of analysis for AChR clustering.

3.3 Analyses of *RAPSN* mutations

3.3.1 Summary of mutations that were investigated

Genetic screening for *RAPSN* mutations was performed by the amplification of the promoter and coding regions of *RAPSN* (exons 1-8) by PCR and amplicons were analysed by Sanger sequencing. This work was done by or referred to the congenital myasthenia service in Oxford, UK.

Eleven novel and potentially pathogenic missense mutations in *RAPSN* coding regions were identified from 11 patients between 2010 and 2015. The mutations identified are summarised in Table 3-1. Amongst these patients, 3 patients were found to harbour the common mutation N88K on their second allele (patients 10, 12 and 13); and 2 patients are compound heterozygous for previously identified non-N88K mutations F185fs (patient 5) and E162K (patient 6). Both mutations were reported to impair the coclustering of rapsyn with AChR in HEK 293 cells (Maselli et al., 2007; Ohno et al., 2002). In particular, F185fs is one of the earliest *RAPSN* mutations, 553ins5, identified to cause CMS (Ohno et al., 2002) where the mutation results in a frameshift for 19 missense amino acids followed by a premature stop codon in TPR5. For the remaining 6 patients, a 2nd mutation has not been found. The identification of a chromosomal microdeletion in *RAPSN* highlights the possibility that heterozygous deletions or duplications in *RAPSN* could be missed by PCR-based sequencing analysis (Muller et al., 2004), which is what has been used by the CMS service. For patient 3, multiplex ligation-dependent probe amplification (MLPA) dosage analysis was performed to search for deletions/duplications of one or more exons within the *RAPSN* gene, but no additional mutation was found. The results for

the other 5 patients are not yet available. However, the MLPA assay has limited sensitivity in detecting partial exon deletion/duplications. It has been suggested that mutations at the intronic sequence element, *Alu*, may mediate genomic rearrangements and rapsyn structure and function (Muller et al., 2004). Therefore further analyses of the genomic DNA of patients may unmask the identity of the mutations on their second alleles.

Mentioned in the introduction, previous studies have shown difficulties in drawing genotype-phenotype correlation in *RAPSN*-CMS. Apart from N88K, homozygous mutations in *RAPSN*-CMS patients are rare, but such cases may provide valuable insights into the correlation between their molecular effects and patient phenotypes. In Oxford, apart from N88K, R91L and V45M were identified to exist in patients in homozygosity, with clinical information on the patients available for analysis. Although their pathogenic properties have already been studied, their effects on AChR clustering were not investigated by the *Rapsyn*-KO model system. The novel mutation A153T was found to be compound heterozygous with F185fs, which is likely to be a null allele. Therefore, particular interest was attached to the results for V45M, N88K, R91L and A153T, and how the phenotypes of patients that do not harbour N88K may vary. The remaining 9 mutations were analysed in cohorts according to when the mutations were identified by the CMS services. In total, the effects of fourteen missense *RAPSN* mutations towards AChR clustering were investigated in this chapter (Table 3-1), and Figure 3-7 shows the relative locations of the mutations in the schematic representation of the rapsyn peptide.

Table 3-1 *RAPSN* mutations identified from 13 patients

Patient	Nucleotide change	Amino acid change	Referred to as	Exon	Domain
1	c.67G>A not known	p.Glu23Lys	E23K	1	TPR1
2	c.133G>A c.133G>A	p. Val45Met p. Val45Met	V45M V45M	1 1	TPR2 TPR2
3	c.221G>A not known	p.Arg74Gln	R74Q	2	Between TPR2 - TPR3
4	c.272G>T c.272G>T	p.Arg91Leu p.Arg91Leu	R91L R91L	2 2	TPR3 TPR3
5	c.457G>A c.549_553dupGTTCT	p.Ala153Thr p.Phe183fs	A153T F183fs	3 3	TPR4 TPR5
6	c.284G>A c.602 G>A	p.Glu162Lys p.Ser201Asn	E162K S201N	2 3	Between TPR4 - TPR5 Between TPR5 - TPR6
7	c.769C>T not known	p.Arg257Trp	R257W	4	TPR7
8	c.791C>T not known	p.Thr264Ile	T264I	5	TPR7
9	c. 821G>A not known	p.Ser274Asn	S274N	5	TPR7
10	c.264C>A c.986A>C	p.Asn88Lys p.His329Pro	N88K H329P	2 7	TPR3 Coiled-coil
11	c.1126C>T not known	p.Arg376Trp	R376W	7	RING-H2
12	c.264C>A c.1159C>T	p.Asn88Lys p.His387Tyr	N88K H387Y	2 7	TPR3 RING-H2
13	c.264C>A c.1195T>A	p.Asn88Lys p.Cys399Ser	N88K C399S	2 8	TPR3 RING-H2

*Nucleotide numbering corresponds to *RAPSN* cDNA sequence, *RefSeq accession: NM_005055/NP_005046*
In Blue are novel *RAPSN* mutations where their pathogenicity have not been confirmed.

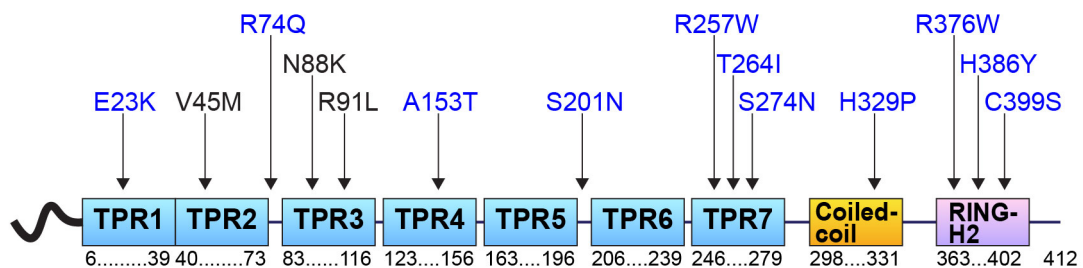


Figure 3-7 Schematic representation of *RAPSN* mutations investigated

The pathogenicity of 14 *RAPSN* mutations was investigated: 11 are novel mutations highlighted in blue, and 3 previously reported mutations confirmed to be pathogenic were also reinvestigated.

3.3.2 Clinical information of patients harbouring the mutations

Clinical information on patients reported was obtained from case note reviews or communications from referring physicians. No information was available for patients 1 and 9. There is limited information for patients 6, 11 and 13 because they were referred to the CMS services from abroad. Therefore the results discussed would be based upon information that was documented from 11 patients. A summary of the clinical features of the patients can be found in Table 3-2.

Ten patients were presented with symptoms at birth, information on patient 6 was taken from interviews retrospectively, and therefore the age at onset is not known. Seven patients presented with ptosis. Facial dysmorphism (patients 4 and 7) and high-arched palate (patients 4 and 12) were present in a few patients. Bulbar symptoms were prominent and affected 8 of the 11 patients, and 6 of these patients required naso-gastric tube feeding. For patients 4 (homozygous R91L) and 5 (A153T/F185fs), gastrotomy was required which suggests they had severe bulbar weaknesses. Six patients had respiratory weaknesses and required invasive assisted ventilation either immediately after birth or during infancy. In particular, patient 9 (T264I/*) required ventilation since birth, but sadly she died at the age of 20 days after extubation. Information on episodic crises was unclear for patients 12 and 14. Hence, 7 of the 9 cases, where information was available, were reported have had at least one episodic worsening precipitated by infection, fever or unknown reasons; and 1 patient required ventilatory support during an episode of pneumonia (patient 13-N88K/H387Y). Patient 11 (N88K/H329P) died at the age of 5 months after a respiratory infection with respiratory insufficiency. Multiple joint contractures were

common amongst this cohort of patients, 7 out of 11 had affected shoulders, elbows, hip, knee, and/or feet; and 3 patients were presented with hypotonia (patients 2, 3, 4). Some of the patients have/had relatives that were affected by similar phenotypic features. Patient 2 (homozygous V45M) had a sister who died at the age of 6 months with similar conditions, and patient 8 (R257W/*) also has a sister who presented with a similar phenotype. The mother of patient 9 (T264/*) had 3 brothers that shared similar symptoms to the patient and like the patient herself, died within the first month of life.

Electromyography (EMG) information was available for 9 patients, and decrement was detected in 3 patients by repetitive nerve stimulation (patient 6- E162K/S201N, patient 12- N88K/H387Y and patient 13- N88K/ C399S). Increased jitter and block were reported from 2 patients who had single fibre EMG at rest (patient 4- homozygous R91L; patient 5- A153T/F185fs). No EMG abnormalities were detected for the remaining 4 patients.

The reported summary on patient phenotypes likely represents the worst scenario for most patients as some clinical features were only transient and patients generally improve with age. Therefore, the information provided indicates the relative severity of clinical course during their early childhood. The follow-up information on treatment responses or long-term disease course was limited. Only 4 patients were known to have had treatment with pyridostigmine prior to genetic diagnosis (patients 2, 4, 6 and 12), and one (patient 6) who was also given 3,4-DAP. Patients 2, 4 and 12 had a good response to pyridostigmine treatment, but neither pyridostigmine nor 3,4-DAP completely resolved the symptoms of patient 6.

Table 3-2 Summary of the clinical features of patients

Patient	Gender	Age at onset	Current age	Mutation analyses	Abnormal EMG	Ptosis	Bulbar symptoms	Respiratory difficulties	Limb weaknesses	Joint contractures	Episodic worsening	Other features
1	No clinical data available			E23K/ *	No clinical data available							
2	M	Birth	8	V45M/V45M	-	+	++	+++	-	+	++	Family history of similar phenotype; Hypotonia
3	M	Birth	3	R74Q/*	-	-	+	-	-	-	+	Hypotonia
4	F	Birth	19	R91L/R91L	+ ^J	+	++ ^G	+++	-	+	++	Facial dysmorphism and high-arched palate
5	M	Birth	7	A153T/Phe185fs	+ ^J	+	++ ^G	+++	-	+	++	
6	M	n/a	~30s	E162K/S201N	+ ^D	-	-	-	+	-	-	Deltoid muscle wasting
7	M	Birth	10	R257W/*	-	+	-	-	-	-	++	Family history of similar phenotype; facial dysmorphism
8	F	Birth	Died at 20 days	T264I/*	-	n/a	++	+++	n/a	+	-	Family history of similar phenotype
9	No clinical data available			S274N/ *	No clinical data available							
10	F	Birth	Died at 5 months	N88K/H329P	n/a	-	++	++	+	+	+	Hypotonia
11	M	Birth	14	R376W/*	n/a	+	-	-	+	+	n/a	
12	M	Birth	28	N88K/H387Y	+ ^D	+	++	+++	+	+	++	High-arched palate
13	M	Birth	8	N88K/C399S	+ ^D	+	+*	-	+	-	n/a	

Mutation analyses: * = 2nd mutation not known; n/a = information not available

Abnormal EMG: +^D = decrement > 10% detected by repetitive nerve stimulation, +^J = increased jitter and block interference pattern in single fibre EMG

Bulbar symptoms: + = mentioned (+* = severity not known), ++ = naso-gastric tube-feeding required, ++^G = gastrostomy required

Respiratory difficulties at birth: + = mentioned, ++ = non-invasive assisted ventilation required; +++ = resuscitation and/or invasive intubation for assisted ventilation required

Episodic worsening: + = occurred once; ++ = occurred more than once

3.3.3 Analyses of AChR clustering of *RAPSN*-EGFP variants

To examine the pathogenicity of the fourteen mutations listed in Figure 3-7, the variants of rapsyn-EGFP were expressed in the *Rapsn*-KO cell system and analysed by an AChR clustering assay. A pre-determined optimal concentration of 1 in 10 FLagrin was applied to differentiated myotubes for 16 hours and AChR clusters were labelled with α -BuTx-594. For each variant, including WT-rapsyn-EGFP, a total of 60 images, from 3 independent experimental repeats, were taken at 20x magnification and analysed. The results are presented as percentages relative to WT.

3.3.3.1 Analyses of *RAPSN* mutations: V45M, N88K, R91L and A153T

The expressions of rapsyn-EGFP variants in *Rapsn*-KO myotubes were confirmed by western blotting analyses, and example images of the agrin-induced AChR clusters formed can be found in Figure 3-8. V45M-rapsyn-EGFP was expressed at a lower level than WT-rapsyn-EGFP, which could affect AChR clustering. Detailed analyses on the effects of V45M towards the expression will be reported in the Chapter 4.

While the number and size of AChR clusters formed on myotubes expressing V45M-, R91L- and A153T- rapsyn-EGFP were clearly reduced compared to cells expressing WT-rapsyn-EGFP, the difference effect of the mutation N88K is more subtle. Hence the images acquired were analysed by both Fiji macro and Volocity analysis to confirm the pathogenic effects of the *RAPSN* mutations and to evaluate if Fiji macro analysis is able to identify subtle differences in the properties of AChR clusters (Figure 3-9).

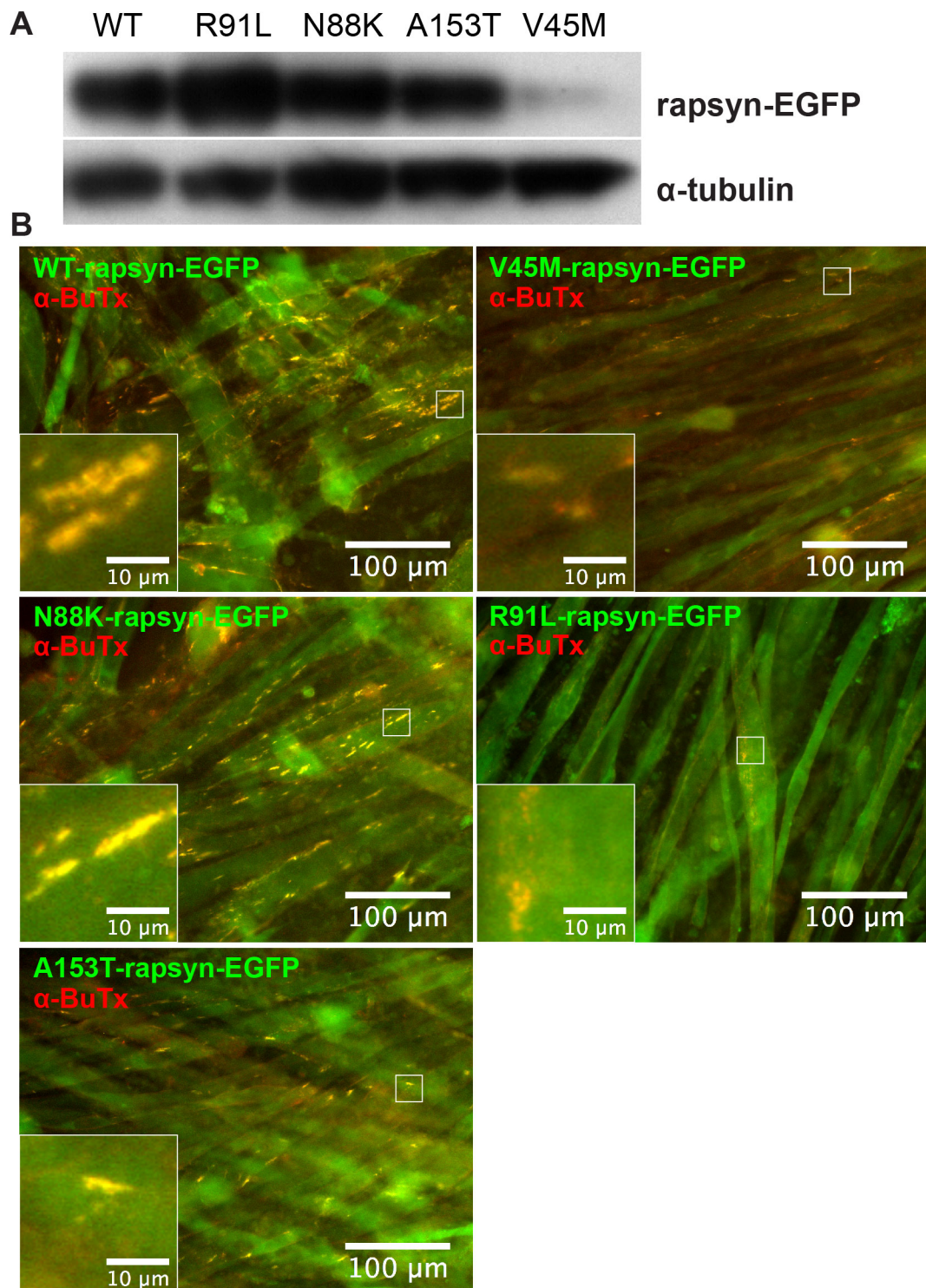


Figure 3-8 Representative microscopic fields from AChR clustering assay for V45M-, N88K-, R91L- and A153T-rapsyn-EGFP

The expressions of rapsyn-EGFP variants were introduced to *Rapsn*-KO cells, and differentiated myotubes were treated with 1 in 10 FLAgrin to induce AChR clustering.

(A) Western blotting using an GFP-antibody confirmed rapsyn-EGFP expression

(B) Representative images of AChR clusters labelled with α-BuTx-594

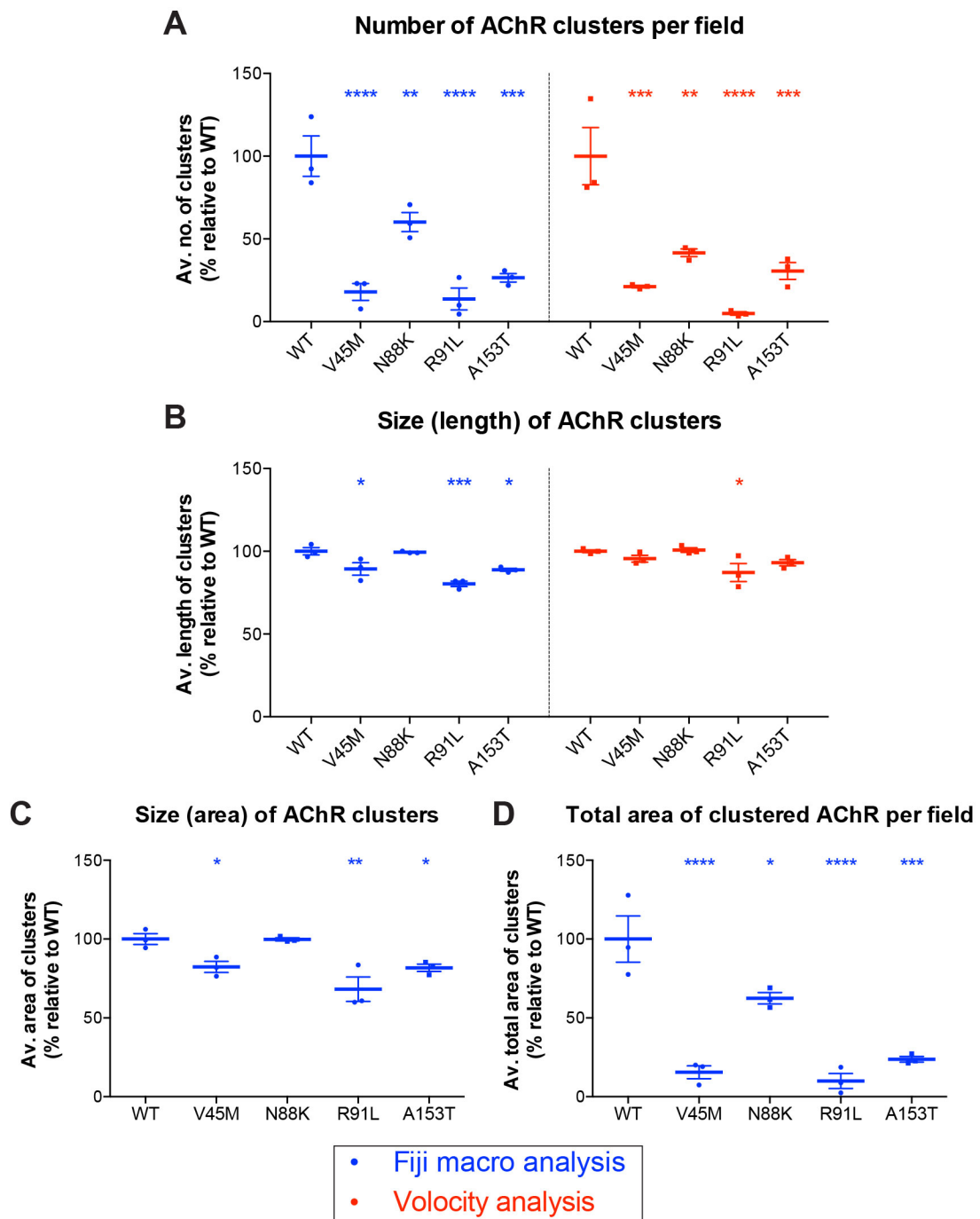


Figure 3-9 Quantified data on AChR clustering by V45M, N88K, R91L and A153T-rapsyn-EGFP

Myotubes expressing WT- or mutant-rapsyn-EGFP were stimulated with FLa-grin for AChR clustering, and AChR clusters were labelled with α -BuTx-594. For each variant, 60 images from 3 experiments were analysed by both Fiji macro and Volocity analysis.

The data points represent the mean of the data from 20 images for each condition, and the results are summarised by overall mean $\pm$ SEM. Asterisks **** p <0.0001; *** p <0.001; ** p <0.01; * p <0.05. One-way ANOVA and Dunnet's multiple comparisons test.

The number of AChR clusters formed per microscopic field on myotubes expressing V45M-, N88K-, R91L- and A153T- rapsyn-EGFP was significantly lower than the number of clusters formed on myotubes expressing WT-rapsyn-EGFP.

According to Fiji macro analyses, the average lengths of AChR clusters formed by R91L-, A153T- and V45M- rapsyn-EGFP were shorter in comparison to AChR clusters formed by WT-rapsyn-EGFP, and N88K did not affect the length of the clusters formed. On the other hand, Volocity analysis only detected shorter AChR clusters formed by R91L-rapsyn-EGFP when compared to WT.

From Fiji macro analysis, the average areas of AChR clusters are similar to the length of AChR clusters. Clusters formed by V45M-, R91L-, A153T- rapsyn-EGFP were significantly smaller than WT-rapsyn-EGFP. The average area of AChR clusters formed by N88K-rapsyn-EGFP was similar to WT.

The results on the total area of clustered AChR per field show a similar pattern to the number of AChR clusters formed. When compared to WT, the total area of AChR clusters formed per field by all of the mutant-rapsyn-EGFP was significantly smaller.

As the *RAPSN* mutations investigated in this section (V45M, N88K, R91L and A153T) reduced the number and total area of the AChR clusters formed, they were considered to be pathogenic mutations.

The relative differences in the number and length of AChR clusters formed by mutant and WT rapsyn-EGFP reported by Fiji macro analysis and Volocity were consistent. To statistically confirm the results from the two methods of analysis are in agreement with each other, a correlation analysis was performed on the raw data (Figure 3-10).

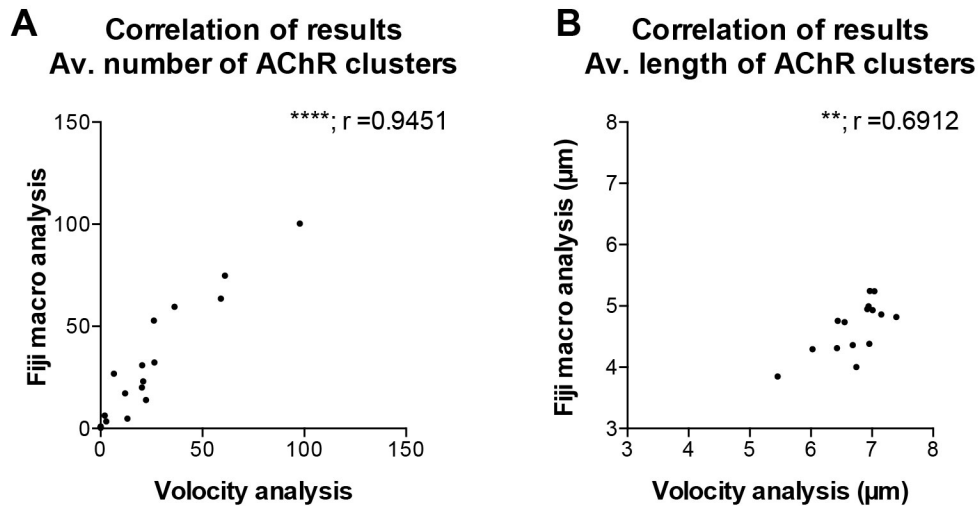


Figure 3-10 Correlation between results from Fiji-macro and Volocity analyses

The results from Fiji macro and Volocity analysis on AChR clustering of WT-, V45M-, N88K-, R91L- and A153T-rapsyn-EGFP were analysed.

The data points represent the mean of the data from 20 images. Pearson correlation coefficient, r , was calculated to confirm the correlation. Asterisks **** $p<0.0001$; ** $p<0.01$.

The Pearson correlation coefficient for the number of AChR clusters quantified is high ($r=0.9351$, $p<0.0001$). The lengths of AChR clusters reported by Fiji macro analysis tends to be shorter than the results from Volocity, hence the measurement for length is less well correlated, but still significant, between the two methods of quantification ($r=0.6912$, $p<0.0043$).

Mentioned previously, the measurement of the length of AChR clusters by Volocity analysis is limited by the technical difficulties in drawing measurement lines in precisely. This is likely to be the reason for the longer lengths of clusters reported. Fiji macro analysis uses the same set of parameters to quantify AChR clusters in an experiment, and this controls for consistency in the analysis between images. In contrast, manual quantification in Volocity is likely to generate variation in results, especially when more than hundreds or even thousands of AChR clusters are to be quantified in an experiment. Consistency in analysis enables higher sensitivity in detecting subtle changes, which is demonstrated in Figure 3-9 **B**. Therefore, it was concluded that Fiji macro analysis is a reliable method that can quantify the properties of AChR clusters formed on myotubes in an objective and consistent manner. As AChR clusters on images are 2-dimensional, area analysis would be used instead of length to quantify the size of the clusters formed; and all of the other AChR clustering experiments described in this thesis were analysed by Fiji macro analysis.

3.3.3.2 Analyses of *RAPSN* mutations: E23K, H329P, R376W and H387Y

Western blotting analysis of the whole cell lysates from myotubes suggests similar protein expression levels of the rapsyn-EGFP variants to WT, and example fields of the agrin-induced AChR clusters formed are shown in Figure 3-11.

E23K, H329P, R376W and H387Y reduced the number and total area of the AChR clusters formed. In addition, the size of AChR clusters formed by H329P- and H387Y- rapsyn-EGFP were smaller than AChR clusters on myotubes expressing WT-rapsyn-EGFP. It was concluded that *RAPSN* mutations E23K, H329P, R376W and H387Y are pathogenic (Figure 3-12).

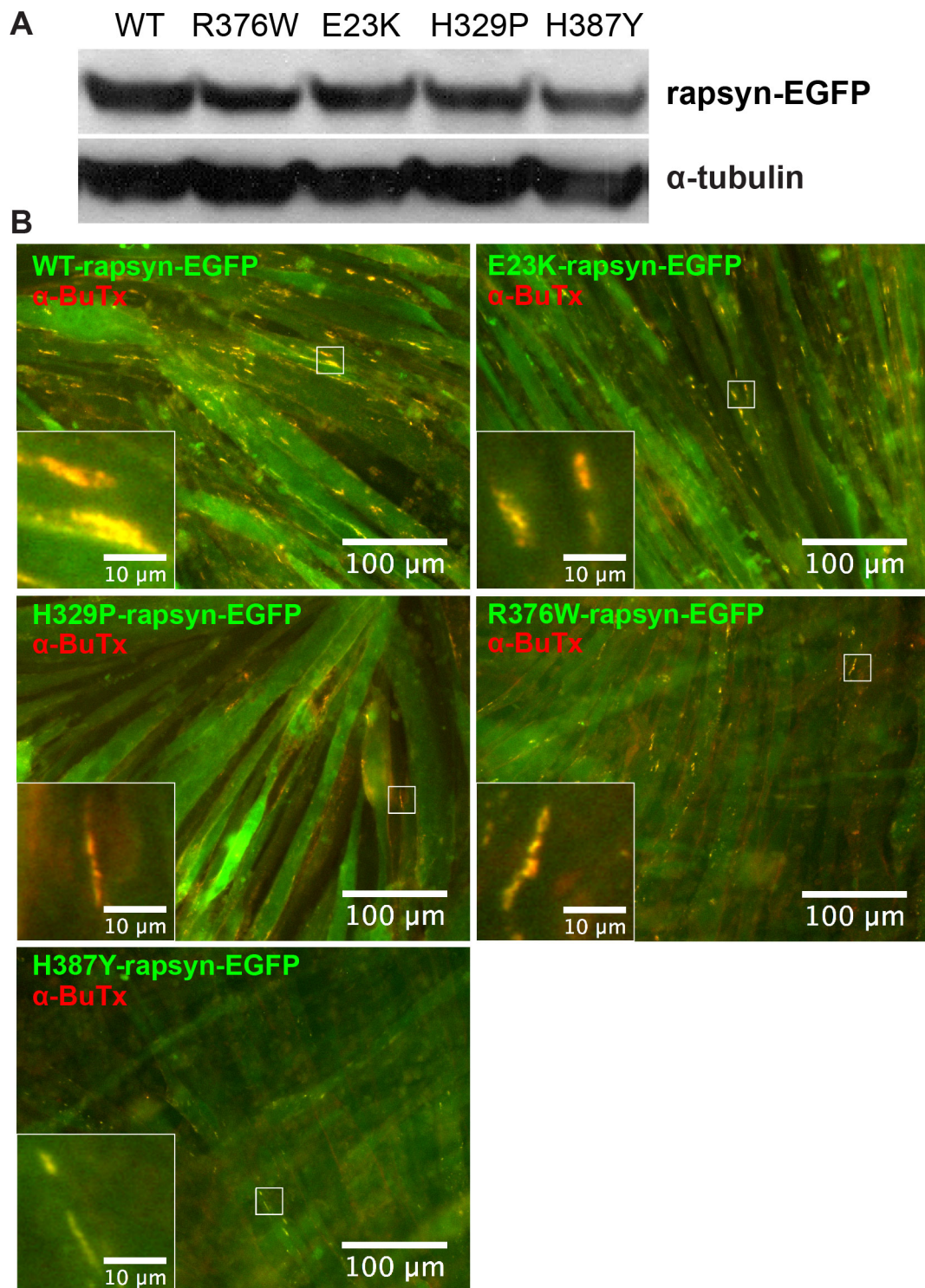


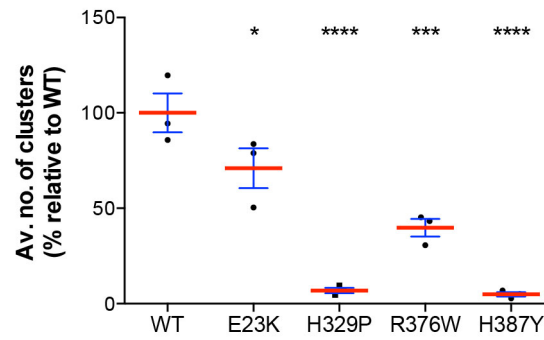
Figure 3-11 Representative images from the AChR clustering assay for E23K-, H329P-, H387Y- and R376W-rapsyn-EGFP

The expressions of rapsyn-EGFP variants were introduced to *Rapsn*-KO cells, and differentiated myotubes were treated with 1 in 10 FLAgrin to induce AChR clustering.

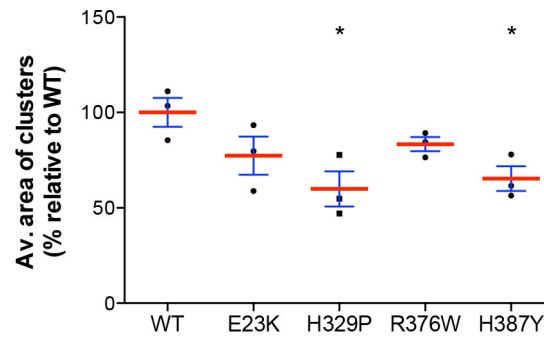
(A) Western blotting using an GFP-antibody confirmed rapsyn-EGFP expression

(B) Representative microscopic fields of AChR clusters labeled with α-BuTx-594

A Number of AChR clusters per field



B Size (area) of AChR clusters



C Total area of clustered AChR per field

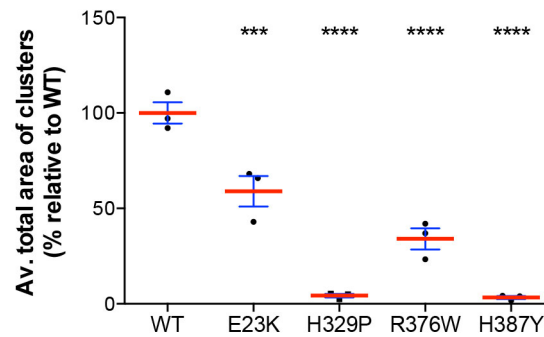


Figure 3-12 Quantified data on the AChR clustering by E23K-, H329P-, H387Y- and R376W-rapsyn-EGFP

Myotubes expressing WT- or mutant-rapsyn-EGFP were stimulated with FLAgrin for AChR clustering, and AChR clusters were labelled with α -BuTx-594. For each variant, 60 images from 3 experiments were analysed by Fiji macro analysis.

Data points represent the mean of the data from 20 images for each condition, and the results are summarised by overall mean $\pm$ SEM. Asterisks **** p <0.0001; *** p <0.001; * p <0.05. One-way ANOVA and Dunnet's multiple comparisons test.

3.3.3.3 Analyses of *RAPSN* mutations: R74Q and R257W

Western blotting analyses of whole cell lysates confirmed similar protein expression levels of rapsyn-EGFP between myotubes expressing the variants. Representative images of agrin-induced AChR clusters are shown in Figure 3-13.

Both mutations R74Q and R257W reduced the number and total area of agrin-induced AChR clusters formed when compared to myotubes expressing WT-rapsyn-EGFP. R74Q also reduced the average size of the AChR clusters formed. Therefore both R74Q and R257W *RAPSN* mutations were concluded to reduce AChR clustering and are pathogenic (Figure 3-14).

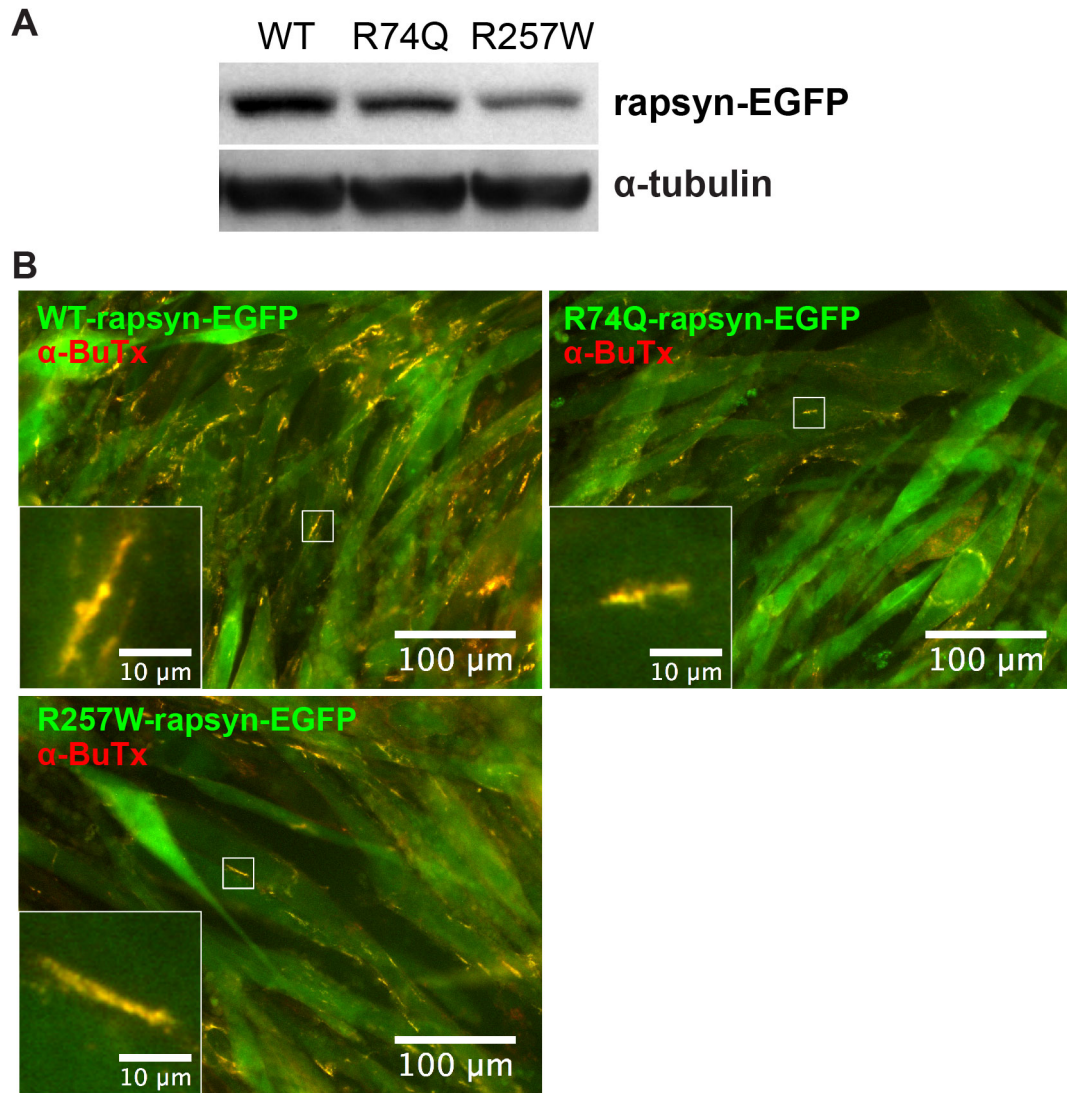


Figure 3-13 Representative images from the AChR clustering assay for R74Q- and R257W-rapsyn-EGFP

The expressions of rapsyn-EGFP variants were introduced to *Rapsn*-KO cells, and differentiated myotubes were treated with 1 in 10 FLAgrin to induce AChR clustering.

(A) Western blotting using an GFP-antibody confirmed rapsyn-EGFP expression

(B) Representative microscopic fields of AChR clusters labelled with α-BuTx-594

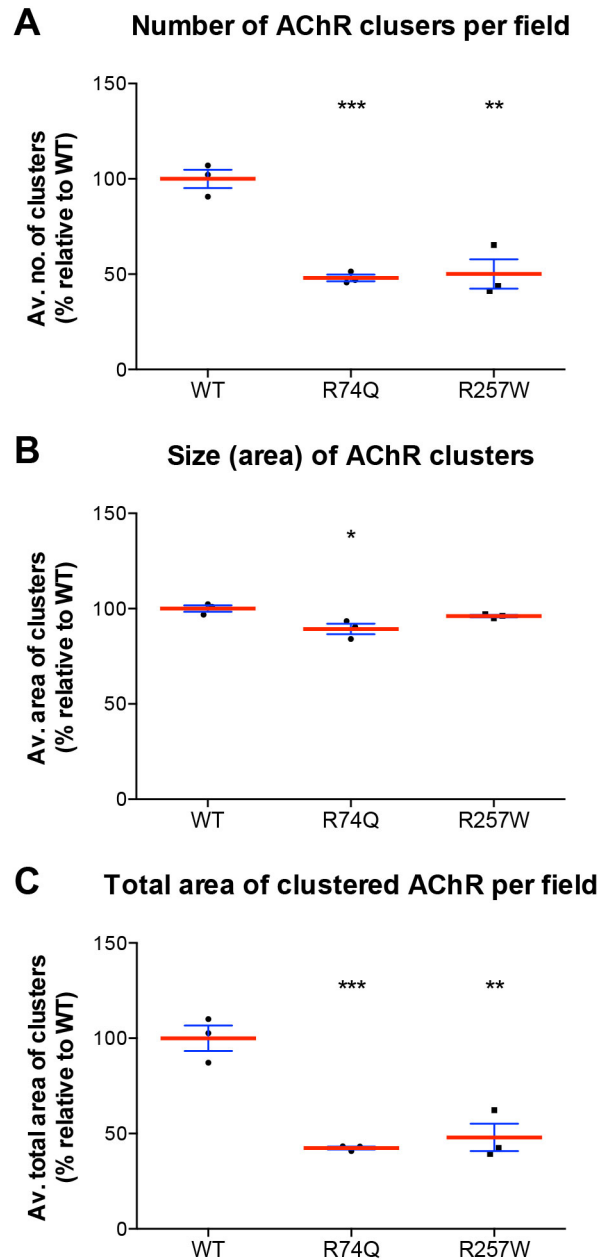


Figure 3-14 Quantified data on the AChR clustering assay for R74Q and R257W-rapsyn-EGFP

Myotubes expressing WT- or mutant-rapsyn-EGFP were stimulated with FLaGrin for AChR clustering, and AChR clusters were labelled with α -BuTx-594. For each variant, 60 images from 3 experiments were analysed by Fiji macro analysis.

Data points represent the mean of the data from 20 images for each condition, and the results are summarised by overall mean $\pm$ SEM. Asterisks **** p <0.0001; *** p <0.001; * p <0.05. One-way ANOVA and Dunnet's multiple comparisons test.

3.3.3.4. Analyses of *RAPSN* mutations: S201N and C399S

Western blotting analysis of whole cell lysate of myotubes showed higher protein expression of S201N-rapsyn-EGFP, and the expression of WT- and C399S-rapsyn-EGFP were similar (Figure 3-15 **A**). Representative fields of agrin-induced AChR clusters formed by the rapsyn-EGFP variants are shown in Figure 3-15 **B**.

The *RAPSN* mutations, S201N and C399S, reduced the number, size and total area of the AChR clusters formed and are therefore concluded as pathogenic (Figure 3-16).

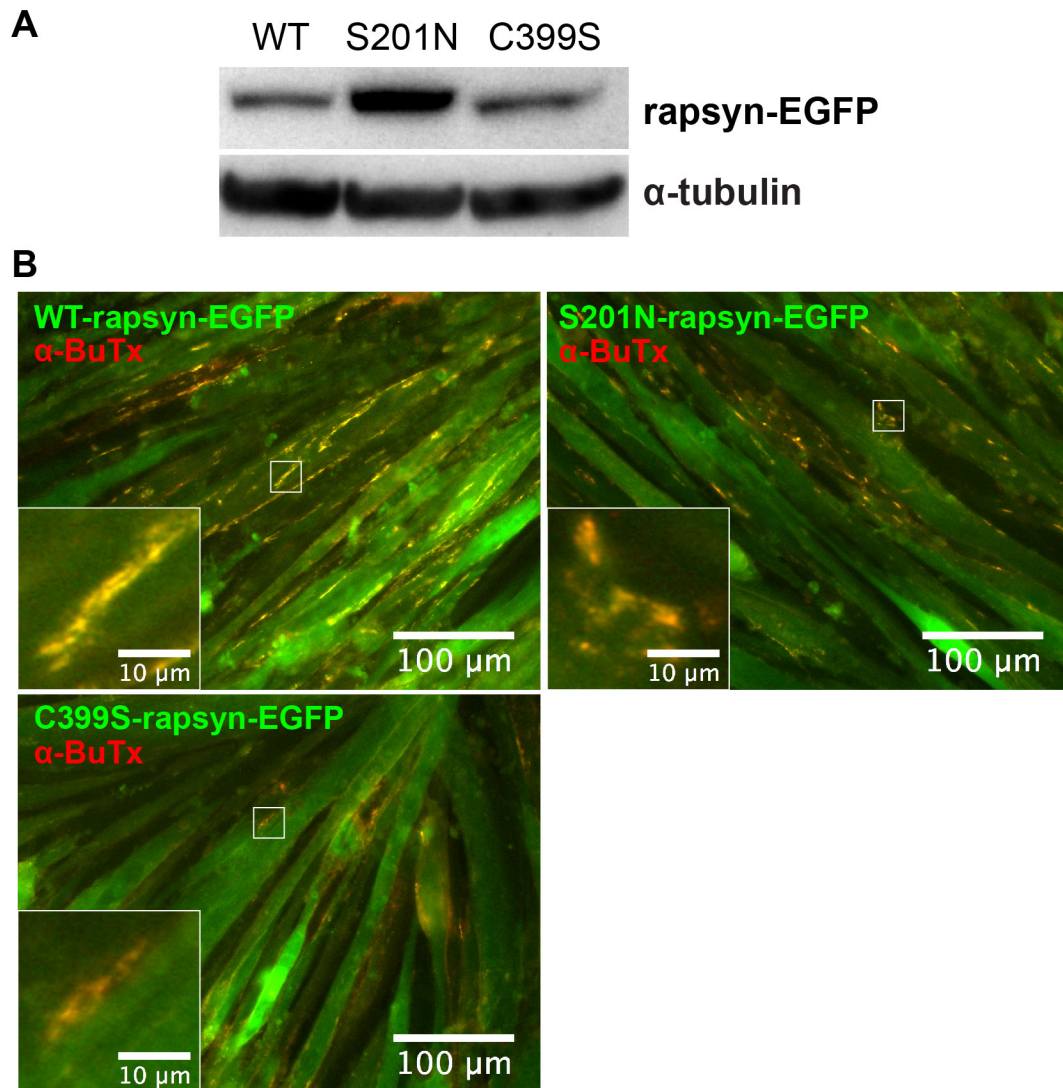


Figure 3-15 Representative images from AChR clustering assay for S201N- and C399S-rapsyn-EGFP

The expressions of rapsyn-EGFP variants were introduced to *Rapsn*-KO cells, and differentiated myotubes were treated with 1 in 10 FLagrin to induce AChR clustering.

(A) Western blotting using a GFP-antibody confirmed rapsyn-EGFP expression

(B) Representative microscopic fields of AChR clusters labeled with α-BuTx-594

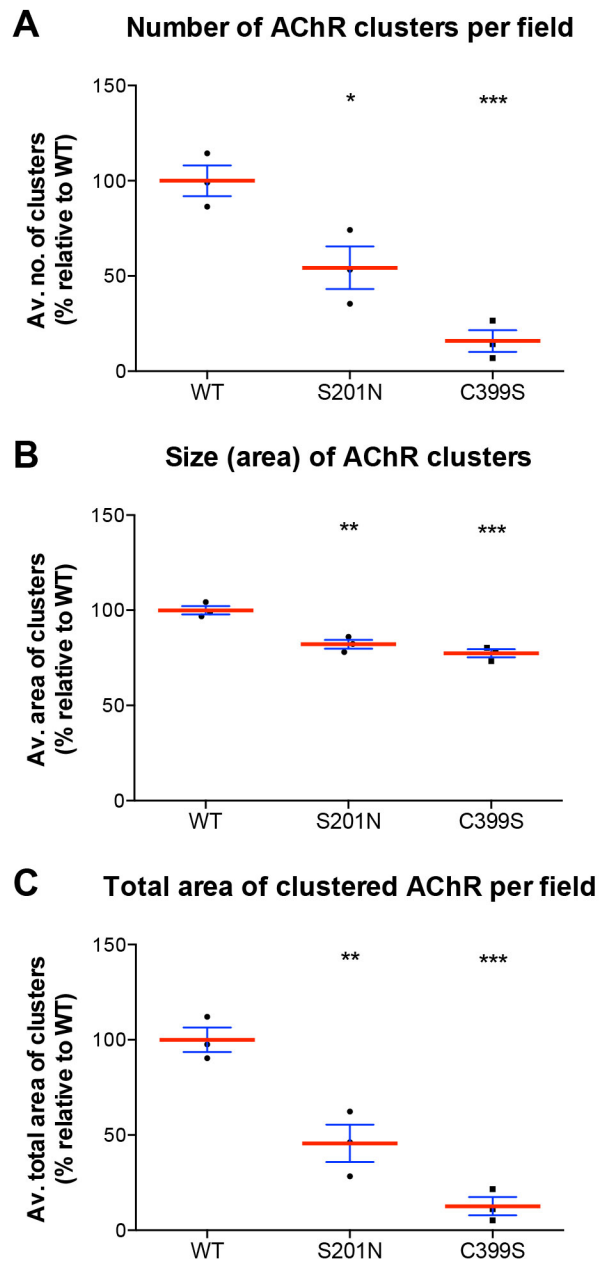


Figure 3-16 Quantified data on the AChR clustering assay for S201N- and C399S-rapsyn-EGFP

Myotubes expressing WT- or mutant-rapsyn-EGFP were stimulated with FLagrin for AChR clustering, and AChR clusters were labelled with α -BuTx-594. For each variant, 60 images from 3 experiments were analysed by Fiji macro analysis.

Data points represent the mean of the data from 20 images for each condition, and the results are summarised by overall mean $\pm$ SEM. Asterisks *** p <0.001; ** p <0.01; * p <0.05. One-way ANOVA and Dunnet's multiple comparisons test

3.3.3.5. Analyses of *RAPSN* mutations: T264I and S274N

The protein expression levels of the rapsyn-EGFP variants were similar, and the representative fields of AChR clusters formed following agrin stimulation are shown in Figure 3-17.

The *RAPSN* mutation, T264I, reduced the number of AChR clusters formed and the total area of clustered AChR per microscopic field, and is therefore is concluded as pathogenic. S274N did not affect the parameters tested in this assay, and is therefore unlikely to be pathogenic (Figure 3-18).

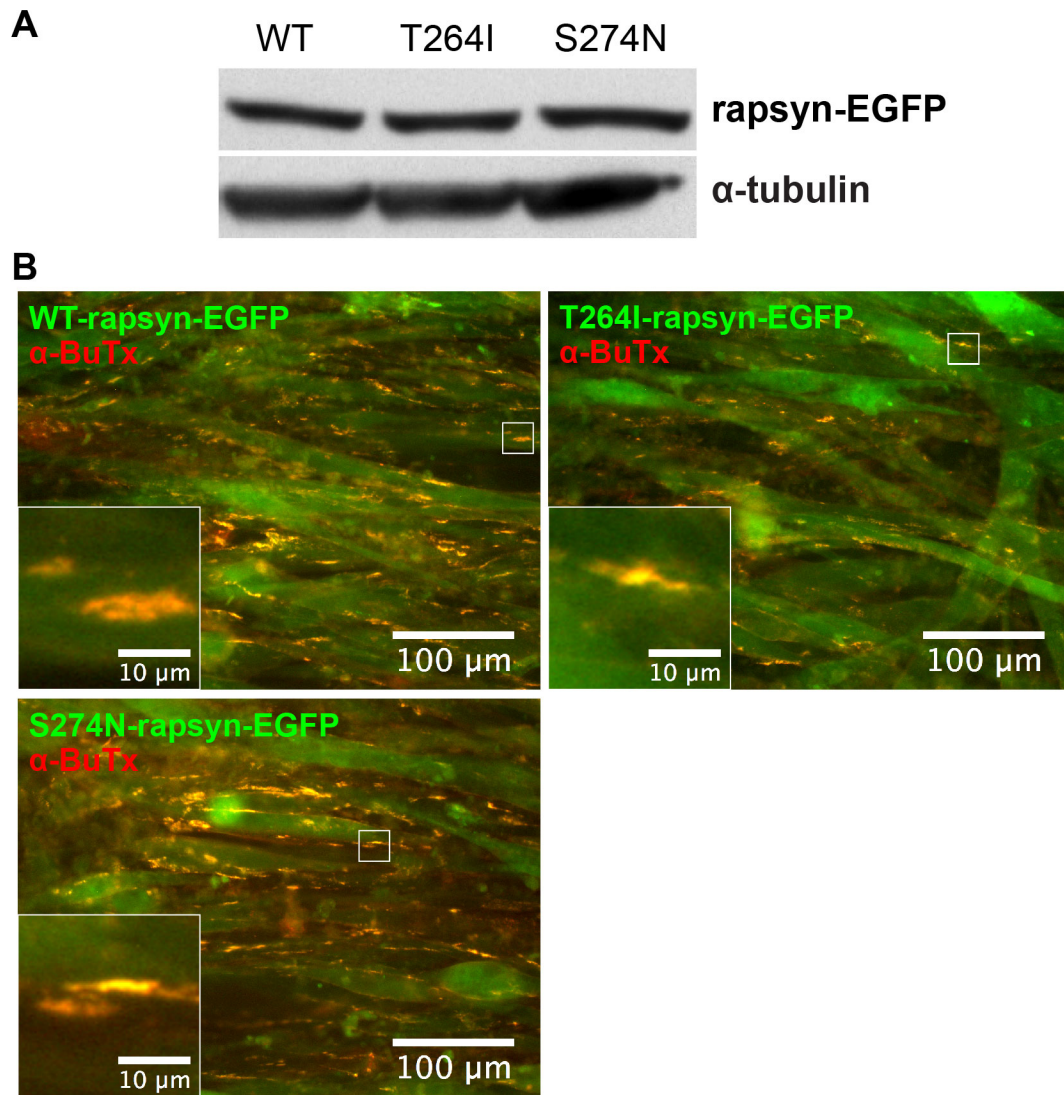


Figure 3-17 Representative images from the AChR clustering assay for T264I- and S274N-rapsyn-EGFP

Expressions of rapsyn-EGFP variants were introduced to *Rapsn*-KO cells, and differentiated myotubes were treated with 1 in 10 FLagrin to induce AChR clustering.

(A) Western blotting using an GFP-antibody confirmed rapsyn-EGFP expression

(B) Representative microscopic fields of AChR clusters labelled with α-BuTx-594

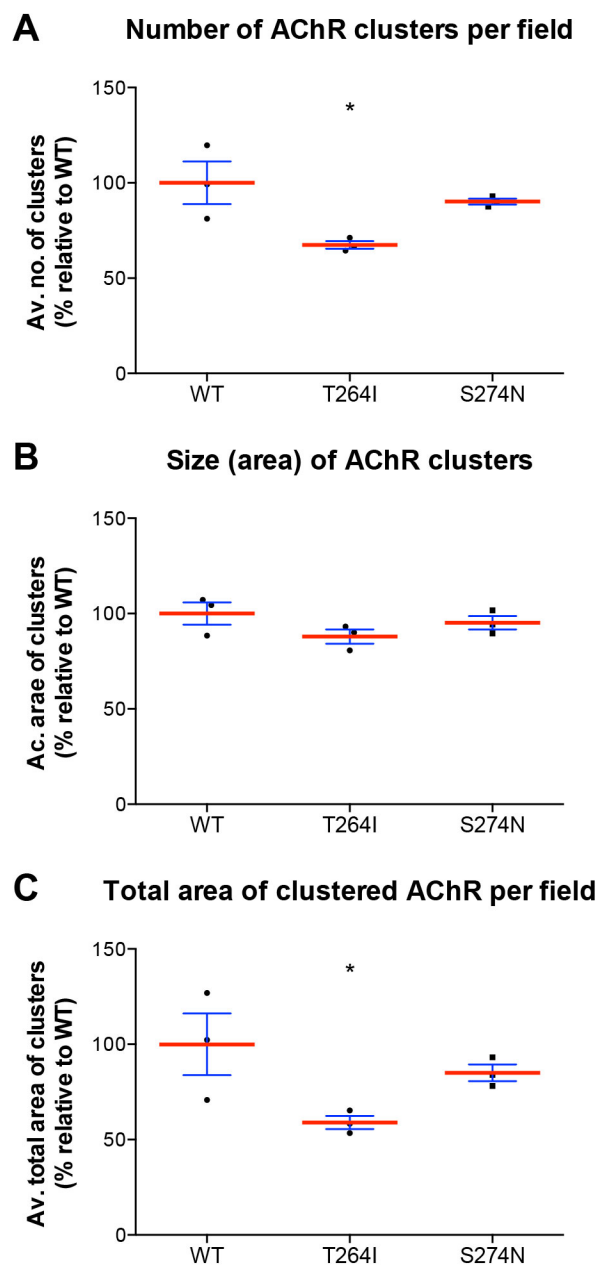


Figure 3-18 Quantified data on the AChR clustering assay for T264I and S274N-rapsyn-EGFP

Myotubes expressing WT- or mutant-rapsyn-EGFP were stimulated with FLAgrin for AChR clustering, and AChR clusters were labelled with α -BuTx-594. For each variant, 60 images from 3 experiments were analysed by Fiji macro analysis.

The data points represent the mean of the data from 20 images for each condition, and the results are summarised by overall mean $\pm$ SEM. One-way ANOVA and Dunnet's multiple comparisons test, asterisk * $p < 0.05$.

3.3.4 Variations in impaired AChR clustering caused by *RAPSN* mutations

When the results from the analysis of the effects of all of the *RAPSN* mutations on AChR clustering are plotted, variations in the reductions of AChR clusters formed can be observed (Figure 3-19). For example, the mutation N88K had a mild effect and only reduced the total area of AChR to ~60% relative to the WT control, and there are mutations such as H329P that almost completely ablated AChR clustering, and reduced the total area of clustered AChR to ~5%.

It was tempting to identify whether there are any correlations between the severities of reduced AChR clustering and clinical phenotype, but the variable phenotypes in *RAPSN*-CMS patients that harbour the same mutations and the sample size presented in the thesis do not allow for accurate conclusions to be made. However, patients with *RAPSN* mutations have been reported in cases of fatal foetal akinesia syndromes (Vogt et al., 2008), and perhaps homozygous cases of the most severe mutations, such as H329P, would be lethal.

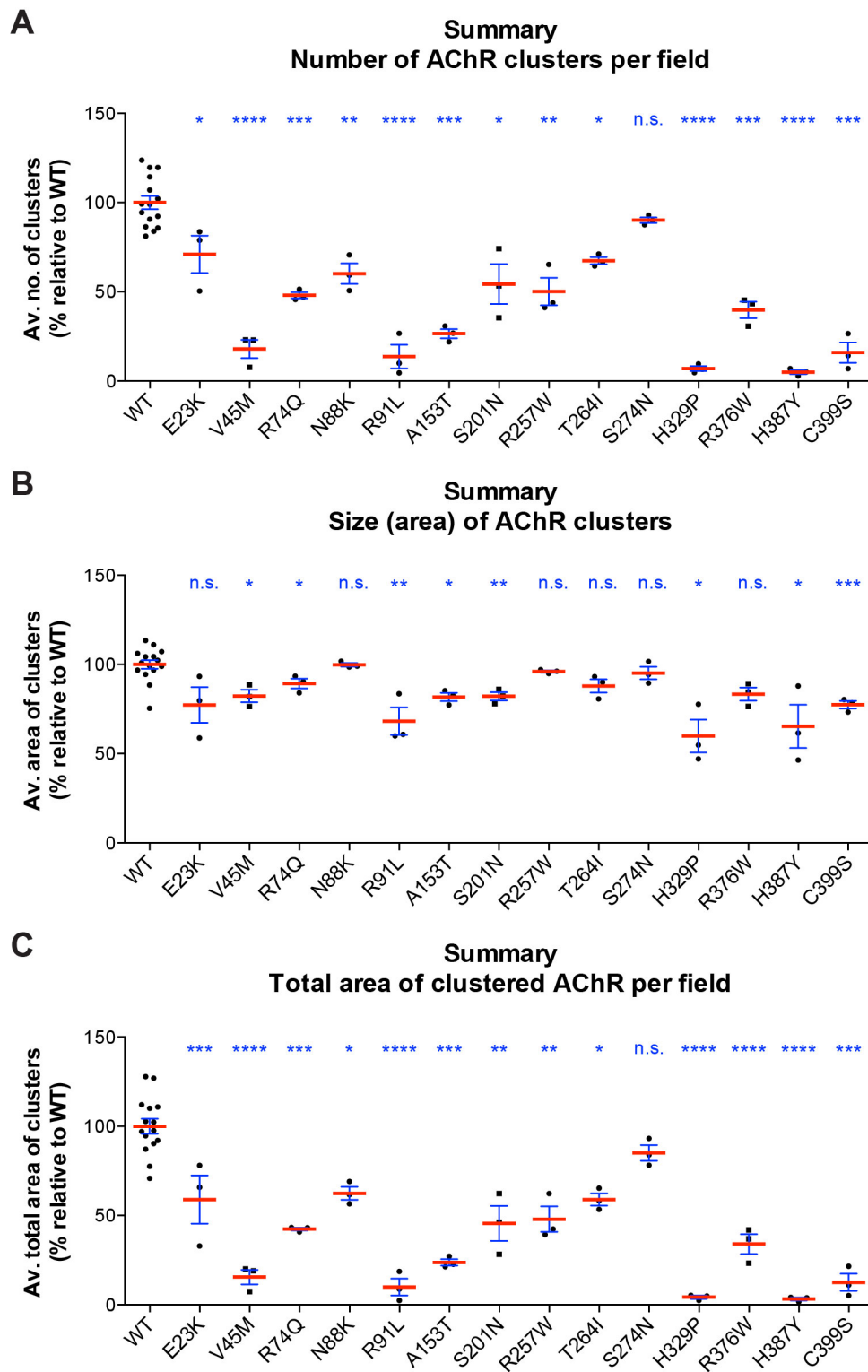


Figure 3-19 Grouped data on the effects of *RAPSN* mutations on AChR clustering

The results from the analysis on the effects of *RAPSN* mutations on AChR clustering are summarised. The relative changes in AChR clustering properties and the statistical analyses are to the respective their originating group of analysis, with the WT data normalised to 100%. Asterisks, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

3.4 Bioinformatics predictions for the functional consequence of mutations in *RAPSN*

The AChR clustering assay is an analysis that requires multiple preparation steps, and it can take up to 4 months before the results are available. Some publications have used prediction analyses to suggest the pathogenicity of novel *RAPSN* mutations (Leshinsky-Silver et al., 2012; Milone et al., 2009). Despite the lack of support from functional analysis, such predictions may be useful for clinicians to make a diagnosis sooner, so that appropriate management plans for patients can be established at an earlier stage. To evaluate the performance of prediction analyses in reflecting the functional effects of amino acid substitutions, I have used the bioinformatics tools, PolyPhen-2 and PROVEAN, to make predictions on the functional consequences of the mutations studied (Table 3-3).

PolyPhen-2 analyses information derived from sequence alignments and protein structural features, and predicts the damaging effects for the enquired single nucleotide change to be benign or, possibly (low confidence)/probably (high confidence), damaging (Adzhubei et al., 2013; Adzhubei et al., 2010). The software PROVEAN can predict consequences for mutations that are not restricted to missense mutations (Choi et al., 2012b). This could be useful for making predictions for other *RAPSN* mutations identified. PROVEAN calculates a delta-alignment score based on the sequence conservation of the mutated residues, and reports binary results that are neutral or deleterious (Choi et al., 2012b). More sophisticated prediction methods are available, but the lack of the crystallographic structure of rapsyn prevents accurate predictions from being made by such analysis methods.

Table 3-3 Prediction on the molecular consequences of amino acid substitutions caused by *RAPSN* mutations

<i>RAPSN</i> mutation	Analysis of AChR clustering	Predictions by PolyPhen-2		Predictions by PROVEAN	
		Score	Prediction*	Score	Prediction**
E23K	pathogenic	0.3620	benign	-1.0800	neutral
V45M	pathogenic	0.9900	probably damaging	-0.5750	neutral
R74Q	pathogenic	0.9490	possibly damaging	-0.9380	neutral
N88K	pathogenic	1.0000	probably damaging	-5.0810	deleterious
R91L	pathogenic	0.9890	probably damaging	-3.5700	deleterious
A153T	pathogenic	0.9690	probably damaging	-2.2570	neutral
S201N	pathogenic	0.5680	possibly damaging	0.1830	neutral
R257W	pathogenic	0.9990	probably damaging	-5.3730	deleterious
T264I	pathogenic	0.2130	benign	-1.8010	neutral
S274N	benign	0.0005	benign	0.3060	neutral
H329P	pathogenic	0.9540	probably damaging	-4.2280	deleterious
R376W	pathogenic	0.9960	probably damaging	-2.2370	neutral-
H387Y	pathogenic	1.0000	probably damaging	-3.5490	deleterious
C399S	pathogenic	1.0000	probably damaging	-5.5130	deleterious

* Benign/ probably damaging = with high confidence; possibly damaging = with low confidence

** Prediction made based on default threshold set at -2.5, i.e. deleterious if score ≤ 2.5

The findings from the AChR clustering assay identified 13 of the 14 mutations studied to be pathogenic. PolyPhen-2 predicted the effect of 11 of these mutations to be probably/possibly damaging. The effect of the 2 mutations missed by PolyPhen-2, T264I and S274N, moderately reduced AChR clustering in the assay performed. PROVEAN only predicted 6 mutations to be potentially deleterious. For the mutation S274N, which AChR clustering results did not identify its damaging effects, both PolyPhen-2 and PROVEAN predictions agreed. Therefore, neither of the prediction methods was able to identify all of the pathogenic mutations confirmed by AChR clustering analysis. This indicates the necessity of functional analysis of novel mutations, and if necessary, PolyPhen-2 may be used to provide preliminary insight into the pathogenicity of novel *RAPSN* mutations.

3.5 Discussion

3.5.1 Quantification of AChR clusters by Fiji macro analysis

The reliability of the computer-automated Fiji macro analysis of AChR clusters is confirmed by its ability to generate data that are consistent with Volocity analysis in 2 different experiments. However, it is important to note that both methods of analysis only provide close estimations regarding the properties of the AChR clusters formed.

The advantage of using Fiji macro analysis to quantify AChR clusters is its ability to objectively quantify the properties of AChR clusters with consistency, with the bonus of it being time efficient. Furthermore, the measurement of clusters in area is not available in the previously used method, Volocity analysis. Hence the macro developed can now be adapted for use in standardised analyses performed by different human observers to yield experimental reports with higher consistency.

The number and total area of AChR clusters formed per microscopic field are better measurements to use to confirm the pathogenicity of the *RAPSN* mutations. Differences in the average size of AChR clusters formed by WT- and mutant-rapsyn-EGFP are often subtle, and therefore may not sensitively indicate the pathogenic properties of the mutations. Nonetheless, this information can be used to understand the underlying mechanisms of reduced AChR clustering. There are other features of AChR clusters, which may also aid understanding of the reduced clustering of AChR, that could be quantified by Fiji, such as their fluorescent intensity, colocalisation with rapsyn-EGFP, or the complexity of clusters. However the images acquired for the experiments performed were not suited for such analysis.

3.5.2 Reductions in AChR clustering confirmed the pathogenic properties of *RAPSN* mutations

Thirteen *RAPSN* mutations (*E23K*, *V45M*, *R74Q*, *N88K*, *R91L*, *A153T*, *S201N*, *R257W*, *T264I*, *H329P*, *R376W*, *H387Y* and *C399S*) were found to cause AChR deficiency *in vitro*, and are therefore concluded to be pathogenic. *S274N* did not reduce AChR clustering. This was supported by benign predictions made by PolyPhen-2 and PROVEAN. A summary on the pathogenicity of the mutations investigated can be found in Table 3-4.

Table 3-4 Summary of the pathogenicity of *RAPSN* mutations

<i>RAPSN</i> mutation*	Functional domain	Total area of clustered AChR per microscopic field (% relative to WT)	Analysis of AChR clustering
<i>E23K</i>	TPR1	58.93	pathogenic
<i>V45M</i>	TPR2	17.88	pathogenic
<i>R74Q</i>	TPR2-3	42.44	pathogenic
<i>N88K</i>	TPR3	60.23	pathogenic
<i>R91L</i>	TPR3	13.71	pathogenic
<i>A153T</i>	TPR4	26.63	pathogenic
<i>S201N</i>	TPR5-6	45.58	pathogenic
<i>R257W</i>	TPR7	47.98	pathogenic
<i>T264I</i>	TPR7	59	pathogenic
<i>S274N</i>	TPR7	85.11	benign
<i>H329P</i>	Coiled-coil	4.25	pathogenic
<i>R376W</i>	RING-H2	34.02	pathogenic
<i>H387Y</i>	RING-H2	3.3	pathogenic
<i>C399S</i>	RING-H2	12.62	pathogenic

*Novel mutations are highlighted in blue

The pathogenic properties of 3 of the mutations studied, V45M, N88K and R91L, have been investigated in previous publications (Cossins et al., 2006; Maselli et al., 2007). The mutant V45M-rapsyn-EGFP was studied by its colocalisation with AChR when overexpressed in HEK 293 cells, and significantly fewer cells than WT were found to display colocalisation of rapsyn and AChR (Maselli et al., 2007). Therefore impaired rapsyn-AChR interaction could be the underlying mechanism for the reduced clustering observed in my results. N88K reduced the number of AChR clusters formed by ~30% (Cossins et al., 2006), and in my results there was a ~40% reduction.

When the results from the AChR clustering assays were aligned according to the nucleotide positions of the mutations, the patterns of increasing reduction in AChR clustering were observed in different regions of *RAPSN*. For example, mutations closer to the C-terminus of *RAPSN*, H329P, H387Y and C399S severely reduced AChR clustering. H329P resides within the proposed coil-coil domain, which is suggested to be essential for rapsyn-AChR interaction (Ramarao et al., 2001); H387Y and C399S are located at the RING-H2 domain which is suggested to be important for rapsyn-dystroglycan interaction (Bartoli et al., 2001). However, the mutation R376W also resides within the RING-H2 region, but had a less marked effect on AChR clustering. Therefore it is not possible to conclude which domain/ residue has more functional importance for AChR clustering without conducting molecular analysis in more depth. Moreover, the unknown influences of the C-terminus EGFP-tag on rapsyn cannot be excluded. Although preliminary experiments (not shown) comparing AChR clustering formed by rapsyn-EGFP and rapsyn did not identify the

obvious effects caused by EGFP, possible subtle changes towards the structure of rapsyn affecting its function cannot be excluded.

It is important to note that the overexpressed levels of rapsyn-EGFP variants in *Rapsn*-KO cells do not reflect the expression of rapsyn variants *in vivo*. A single western blotting analysis of lysates of cultures myotubes was performed for each AChR clustering assay to confirm that the pathogenic effects of *RAPSN* mutations observed are not due to the variable expression levels of rapsyn-EGFP, a feature that in this case depends on the infection efficiency of retroviruses. Therefore, the results cannot be used to indicate the effect of mutations on protein expression.

While the results presented suggest the pathogenic properties are *RAPSN* mutations, it might be informative to study AChR clustering on cells with physiological levels of rapsyn expression. The recently established CRISPR/Cas9 mutagenesis technique (Ran et al., 2013b) can be adapted to introduce specific *RAPSN* mutations in C2C12 cells to provide a modified model system to study AChR clustering. If conditions were optimised, the expression levels of rapsyn (without EGFP-tag) in the C2C12 cells could more closely mimic *in vivo* conditions. Details on the feasibility of CRISPR/Cas-9 mutagenesis in C2C12 cells will be discussed in Chapter 5.

3.5.3 Functional analysis is necessary to determine the pathogenic properties of *RAPSN* mutations

The comparison between the results from the assay on AChR clustering and *in silico* predictions made on the consequences of *RAPSN* mutations by PolyPhen-2 and PROVEAN demonstrated that the functional assay is more sensitive in detecting pathogenicity. PolyPhen-2 predicted pathogenicity for most of the *RAPSN* mutations found to impair AChR clustering (11/13); but PROVEAN was less sensitive and only predicted 6 of the mutations to have deleterious effects. One mutation did not affect AChR clustering, and neither of the prediction tools suggested otherwise. However, the current data set does not allow an evaluation of the specificity of predictions, i.e. the occurrences of false negative to be made.

The predictions made by PolyPhen-2 and PROVEAN are both based on the multiple sequence alignments (Adzhubei et al., 2013; Choi et al., 2012b), but features of the algorithm-generated sequence alignments, e.g. the number of sequences aligned, are likely to be different. The accuracy of predictions on protein functionalities is dependent upon the algorithm used to generate the sequence alignments (Hicks et al., 2011). PolyPhen-2 takes into account the available information on the structure of the protein (Adzhubei et al., 2013), which may explain its higher sensitivity in predicting the damaging effects of mutations. An adjusted threshold score may improve the sensitivity of predictions made by PROVEAN (Choi and Chan, 2015), but it would require further analysis of a large sample group to identify a suitable PROVEAN score as the cutoff to make predictions for *RAPSN* mutations. Finally, as current knowledge on the structure of rapsyn is limited, predictions generated by any *in silico* tools should always be treated with caution.

Chapter 4

The underlying mechanisms of *RAPSN* mutations in causing AChR deficiency

4.1 Introduction

The effects of *RAPSN* mutations on AChR clustering have been studied extensively, but little is known about the underlying mechanisms that cause AChR deficiency. One publication investigated the functional effects of *RAPSN* mutations, and identified that a single mutation can cause AChR deficiency through different mechanisms (Cossins et al., 2006), although the effects are likely to be interdependent on one another. From studying 5 *RAPSN* mutations, the mechanisms reported were reduced rapsyn protein stability, impaired rapsyn-rapsyn aggregation, impaired AChR clustering, and the instability of AChR clusters formed (Cossins et al., 2006). To date, no specific mechanisms of missense mutations in *RAPSN* have been identified that explain the variations in the severity of phenotype in patients. Mutations at the promoter E-box region of *RAPSN*, which are associated with facial dysmorphism, reduce the expression of rapsyn mRNA (Ohno et al., 2003). It is important to understand the disease biology to improve management or treatment for patients. In particular, unpredictable respiratory crises are frequently life-threatening.

With limited knowledge on the pathogenic mechanisms of *RAPSN* mutations, studies on the functional roles of rapsyn at the NMJ may provide an insight into this. In cell culture and animal models, up-regulation of rapsyn expression, which increases the ratio of rapsyn to AChR (Gervasio and Phillips, 2005), metabolically stabilises the

level of AChR (Gervasio et al., 2007; Wang et al., 1999). Direct interactions between the TPR domains of rapsyn and AChR were suggested to be essential for this function of rapsyn (Wang et al., 1999). It has been demonstrated in rat muscles that the overexpression of rapsyn increased the expression of AChR in both synaptic and extra-synaptic regions of the muscle membrane (Martinez-Martinez et al., 2007). On the other hand, down-regulation of rapsyn by RNA interference *in vivo* led to a reduction in the protein levels of rapsyn, and a loss of AChR at the NMJ (Martinez-Martinez et al., 2009). The evidence is consistent with a crucial role of rapsyn in stabilising AChR at the postsynaptic membrane of the NMJ, which may be dependent on the amount of rapsyn available for interactions with the receptors.

Disrupted regulation of the turnover of rapsyn attenuates AChR clustering *in vivo*, with AChR clusters becoming fragmented and reduced in size (Luo et al., 2008). This suggests rapsyn is also implicated in the maintenance of AChR clusters. One of the mechanisms involved in the establishment and maintenance of the AChR clusters on the postsynaptic membrane is the balance between the actions of agrin and acetylcholine (ACh). Neural agrin induces AChR clustering and stabilises AChR expression by MuSK signalling (Misgeld et al., 2005), and ACh disassembles AChR clusters via cyclin-dependent kinase 5 (Cdk5) signaling (Lin et al., 2005). The disassembly of AChR clusters requires calpain, a calcium-dependent protease that is involved in the Cdk5 pathway (Chen et al., 2007). It was found that rapsyn interacts with calpain, and disrupted rapsyn-calpain interaction enhanced carbachol (an AChR agonist) induced dispersion of AChR clusters *in vitro* (Chen et al., 2007).

Rapsyn has been suggested to be involved in stabilising AChR by interacting with other proteins enriched at the NMJ. Via its coiled-coil domain, rapsyn anchors protein kinase A (PKA) type I at the postsynaptic region of the muscle membrane, and this was found to stabilise AChR turnover as well as the structure of AChR clusters (Choi et al., 2012a). In addition, phosphorylation of MuSK by the protein kinase CK2, a serine/threonine kinase that rapsyn interacts with, was also found to be important for the stability of AChR clusters (Herrmann et al., 2015). Furthermore, rapsyn interacts with β -dystroglycan (Bartoli et al., 2001), and the dystroglycan complex containing α - and β -dystroglycan are important for AChR stabilisation (Jacobson et al., 2001). AChR clusters formed on dystroglycan-null myotubes are more susceptible to disassembly (Jacobson et al., 2001). The precise roles of rapsyn in these reported pathways have yet to be determined.

RAPSN-CMS patients are more severely affected at a young age, and the frequency and severity of the crises in *RAPSN*-CMS usually diminish during childhood and are rare after the age of 7 years (Finlayson et al., 2013). As both muscles and the NMJ mature, the stability of AChR and AChR clusters increases (Sanes and Lichtman, 2001; Yampolsky et al., 2010). *RAPSN* mutations N88K and L361L reduced the stability of AChR clusters on myotubes (Cossins et al., 2006). In my results, although at a reduced level, N88K-rapsyn-EGFP clearly formed many well-defined AChR clusters. It may be that the impaired stability of AChR clusters due to *RAPSN* mutations has a bigger impact during early life where the structure and stability of the NMJ are more vulnerable to the disruptions provoked by external stimuli.

4.1.1 Aims

This chapter aims to gain insights into the underlying pathogenic mechanisms of *RAPSN* mutations and how this may translate into patient phenotype. The *RAPSN* mutations V45M, N88K, R91L and A153T were investigated (Figure 4-1).

Over 90% of *RAPSN*-CMS patients are either homozygous or on one allele of a compound heterozygous with the N88K mutation. V45M, R91L and A153T were chosen for investigation because they have been identified in patients in the absence of the N88K mutation. It was therefore considered that they may shed light on the ways in which dysfunction of *RAPSN* can cause CMS.

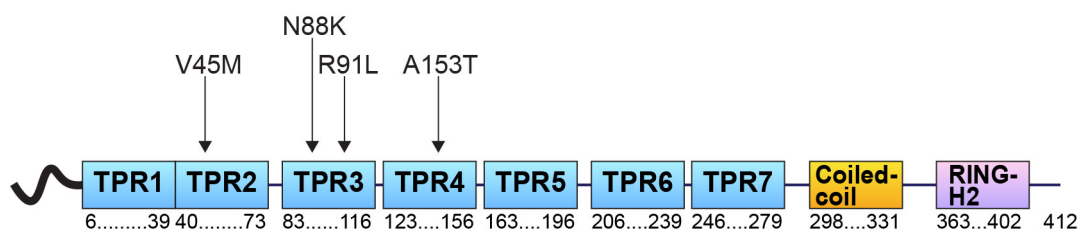


Figure 4-1 *RAPSN* mutations that were investigated for their pathogenic mechanisms

4.2 Effects of *RAPSN* mutations on the protein levels of rapsyn-EGFP

4.2.1 Steady state overexpression of rapsyn-EGFP variants

AChR deficiency can be secondary to a reduced level of rapsyn expression. Constructs encoding cDNA of WT- or mutant-rapsyn-EGFP were transfected into TE671 (human muscle) or HEK (human embryonic kidney) 293 cells (n=3 for both) together with the transfection efficiency marker pcDNA3.1-mCherry. Mock transfection controls, where only pcDNA3.1-mCherry was transfected into cells, were also performed. 48 hours after transfection, lysates were prepared and resolved by western blotting using antibodies against GFP, mCherry and α -tubulin. The ratios of rapsyn-EGFP: mCherry were obtained by densitometry analysis, and the results were normalised to the expression levels of WT-rapsyn-EGFP (Figure 4-2).

In comparison to WT, the V45M mutation significantly reduced the expression of rapsyn-EGFP in TE671 cells, and in HEK 293 cells. The mutation N88K also reduced the levels of overexpressed rapsyn-EGFP in TE671 and in HEK 293 cells, but the difference was only statistically significant in HEK 293 cells. R91L and A153T did not affect the protein levels of rapsyn-EGFP.

Thus V45M severely reduced the steady-state expression of rapsyn-EGFP, while N88K had a modest effect.

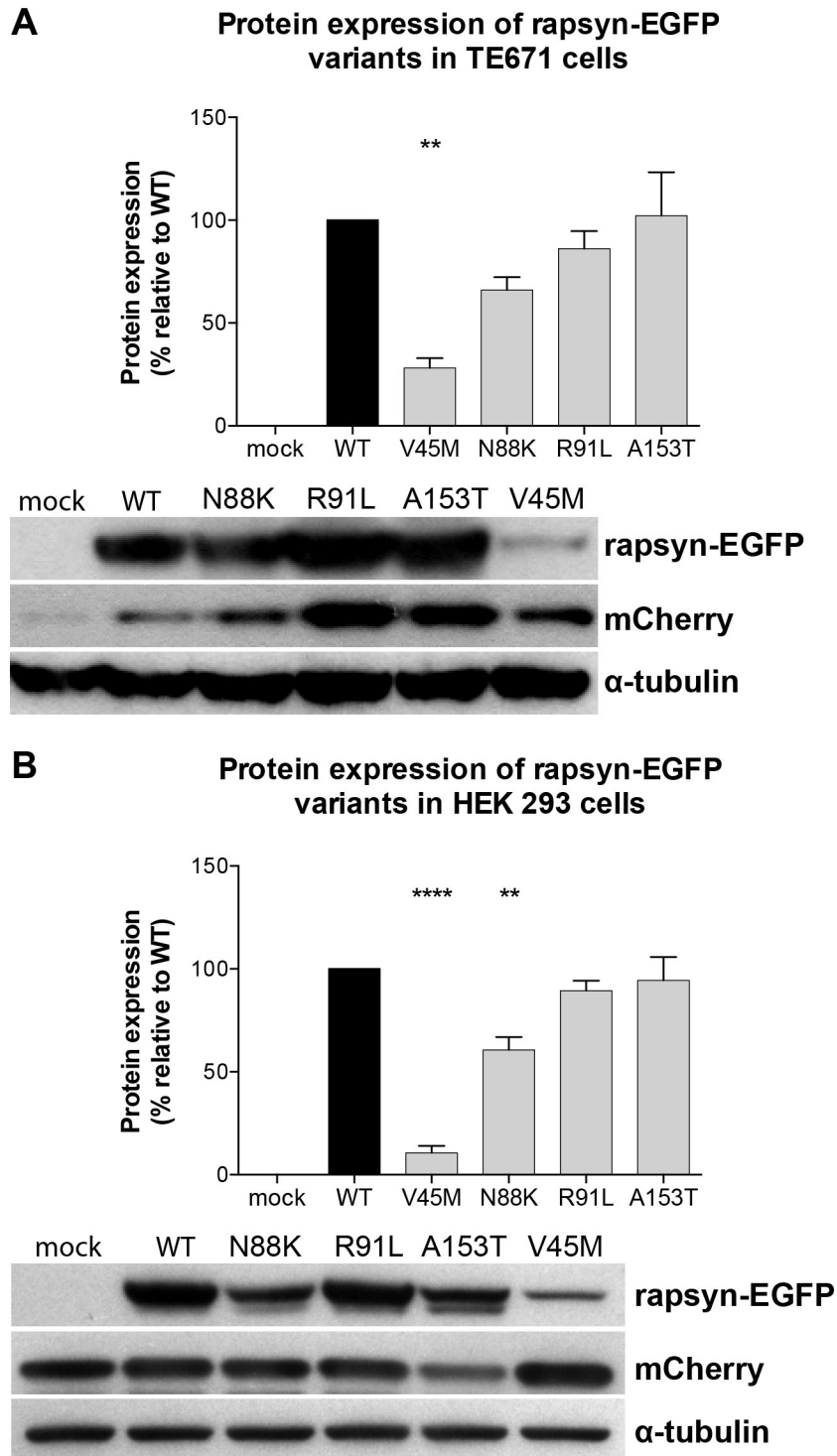


Figure 4-2 Overexpression of rapsyn-EGFP in transfected TE671 and HEK 293

TE671 (A) and HEK 293 cells (B) were transiently transfected with cDNA of WT- or mutant-rapsyn-EGFP, together with a transfection efficiency control, mCherry. 48 hours post-transfection, lysates were prepared and resolved by western blotting using antibodies against GFP, mCherry and α -tubulin. The results (ratio of rapsyn-EGFP: mCherry), $n=3$, are presented as percentages $\pm$ SEM relative to WT. One-way ANOVA and Dunn's multiple comparison test: Asterisks **** $P<0.0001$; ** $p<0.01$.

4.2.2 Stability of rapsyn-EGFP variants

A cycloheximide (CHX) chase assay was performed (n=3). CHX is an inhibitor of protein synthesis in eukaryotic cells. In this assay, variants of rapsyn-EGFP were transfected into HEK 293 cells, and 36 hours after transfection, CHX (final concentration of 20µg/ml in culture medium) was introduced to cells to inhibit protein synthesis. Subsequently, lysates were prepared at 0, 2, 4, 8, 16 and 24 hours after CHX treatment and analysed by western blotting. Quantified results on the relative expression, the ratios of rapsyn-EGFP: α -tubulin, were normalised to the initial expression of the corresponding rapsyn-EGFP variant at 0 hours (Figure 4-3).

The expression of WT-rapsyn-EGFP reduced steadily, and after 24 hours of CHX treatment, to 75.15% SEM $\pm$ 10.715 of its initial expression level. Only 2 hours after the addition of CHX, the relative levels of V45M-rapsyn-EGFP were found to be significantly less than WT. The reduction in expression continued with time, and after 24h of CHX, V45M-rapsyn-EGFP became difficult to detect. The mutations N88K, R91L and A153T also resulted in the reduced expression of rapsyn-EGFP after CHX treatment, and by 24 hours, the relative levels of N88K-, R91L-, and A153T- rapsyn-EGFP were significantly less than WT.

Thus all of the mutations investigated affect the stability of rapsyn-EGFP. While the impacts of N88K, R91L and A153T are relatively mild, the clear-cut effect of V45M explains the reduced protein expression in its steady state.

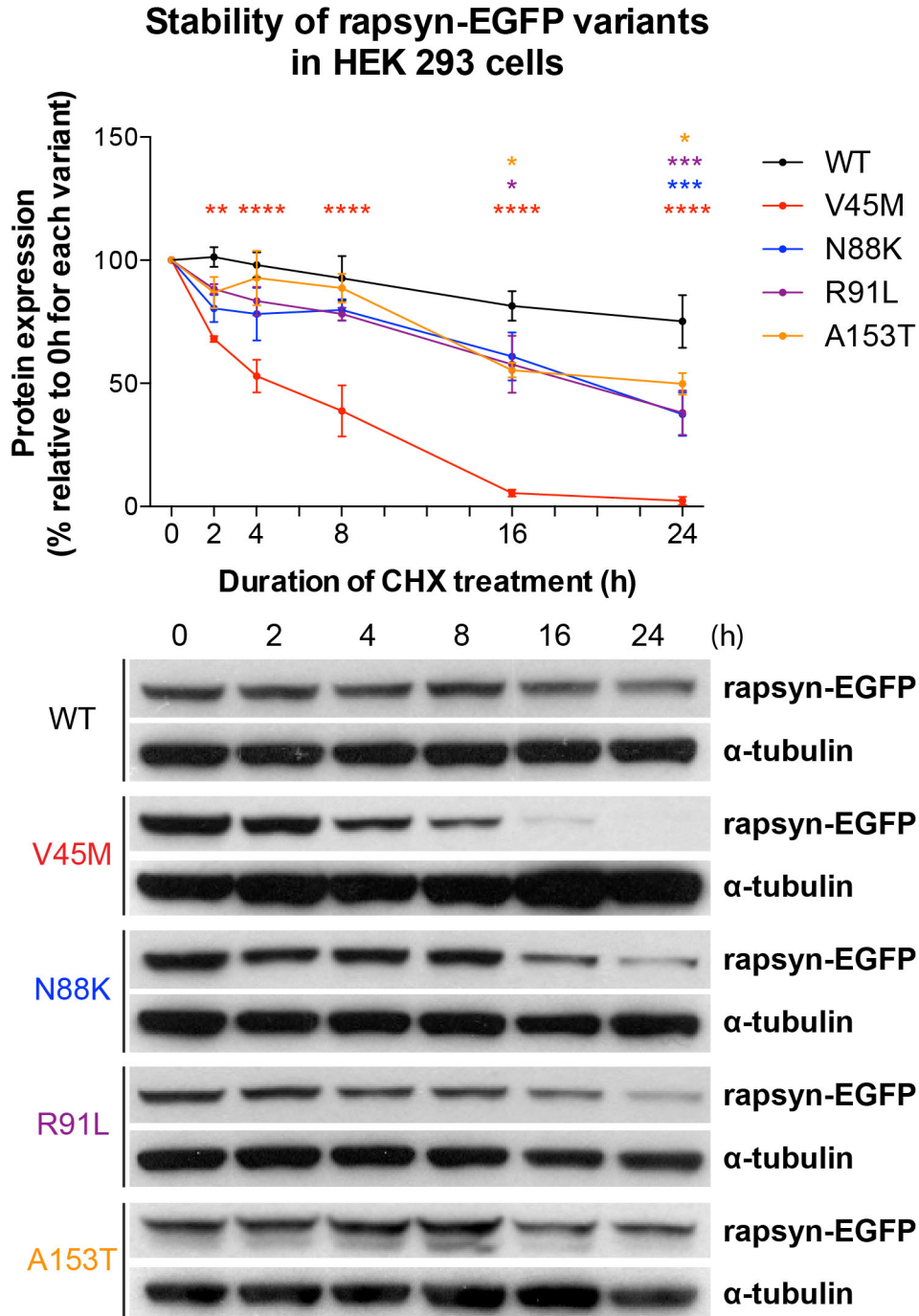


Figure 4-3 Effects of *RAPSN* mutations on the stability of protein expression

HEK 293 cells were transfected with rapsyn-EGFP variants, and 36 hours after transfection, cycloheximide (CHX) was introduced to the transfected cells at the final concentration of 20µg/ml. Cell lysates were harvested at 0, 2, 4, 8, 16 and 24 hours after the addition of CHX, and analysed by western blotting using antibodies against EGFP and α-tubulin. The expression level at 0h was considered to be 100%, hence the ratio of rapsyn-EGFP: α-tubulin were normalised to 0h of each respective variant, and presented as mean percentages ±SEM. Two-way ANOVA with Dunnett's multiple comparisons tests: asterisks ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05

4.3 Effects of *RAPSN* mutations on the levels of AChR on myotubes surfaces

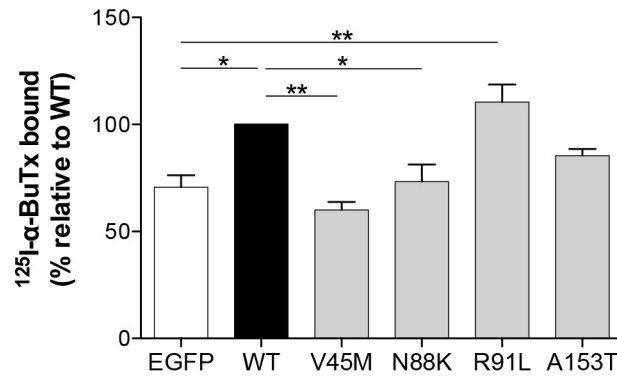
Rapsyn metabolically stabilises AChR on cell surface, and the TPR regions of rapsyn are thought to be essential for this stabilising effect (Wang et al., 1999). To determine if the mutations in *RAPSN* affect the levels of AChR on membrane surfaces, a ^{125}I - α -BuTx binding assay was performed on *Rapsn*-KO myotubes expressing EGFP, WT- or mutant-rapsyn-EGFP. In addition, the assay was performed on myotubes that were either untreated (n=3) or stimulated by agrin for 16 hours (n=6). The quantified surface expressions of AChR, measured by the radioactively labeledled ^{125}I - α -BuTx, are presented as percentages relative to the expression of AChR on myotubes expressing WT-rapsyn-EGFP (Figure 4-4).

Surface expression of AChR was present on *Rapsn*-KO myotubes expressing EGFP, and this level of expression was lower than myotubes expressing WT-rapsyn-EGFP or R91L-rapsyn-EGFP. The levels of AChR on myotubes expressing V45M-, N88K- and A153T-rapsyn-EGFP did not differ from myotubes expressing EGFP.

When compared to myotubes expressing WT-rapsyn-EGFP, the levels AChR on the surfaces of V45M- and N88K- rapsyn-EGFP expressing cells were reduced. The A153T mutation also reduced the levels of AChR, but its effect was modest, as only the difference measured on agrin-treated myotubes was statistically significant. R91L did not affect the surface expression of AChR when compared to WT.

Thus the results suggest that the *RAPSN* mutations V45M, N88K and A153T reduce the steady-state expression of cell surface AChR when compared to WT.

A AChR expression on cell surface of untreated myotubes



B AChR expression on cell surface of agrin-treated myotubes

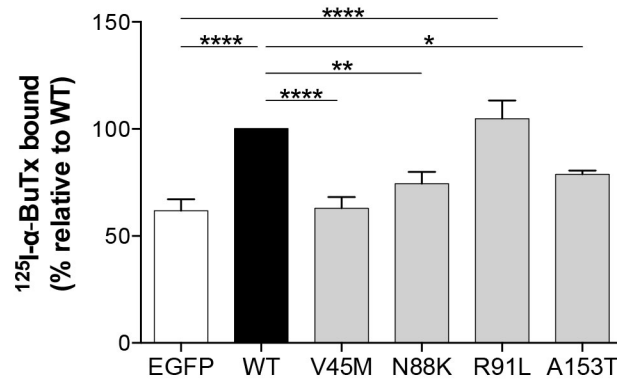


Figure 4-4 $^{125}\text{I-}\alpha\text{-BuTx}$ binding to the surfaces of myotubes expressing EGFP, WT- or mutant-rapsyn-EGFP variants

Rapsn-KO cells expressing EGFP-, WT- or mutant- rapsyn-EGFP were differentiated into myotubes, and the levels of cell surface expression of AChR were measured by $^{125}\text{I-}\alpha\text{-BuTx}$ binding assay.

The results for **(A)** untreated $n=3$ and **(B)** agrin-treated $n=6$ myotubes were normalised to the levels of $^{125}\text{I-}\alpha\text{-BuTx}$ binding to the surfaces of myotubes expressing WT-rapsyn-EGFP. Each bar represents the mean percentages of $^{125}\text{I-}\alpha\text{-BuTx}$ bound to myotubes surfaces $\pm$ SEM. One-way ANOVA with Dunnett's multiple comparisons test, asterisks **** $p<0.0001$, ** $p<0.01$ and * $p<0.05$.

4.4 Stability of AChR clusters formed by rapsyn-EGFP variants

The previous experiments have demonstrated that *RAPSN* mutations may affect both levels of rapsyn-EGFP and cell surface AChR. Next I investigated the stability of AChR clusters. Carbachol is an AChR agonist that destabilises and disrupts AChR clusters at 100 μ M (Bloch, 1986). As such, studying the carbachol induced AChR cluster loss on cultured myotubes can potentially be used to investigate the effect of *RAPSN* mutations on the stability of agrin-induced AChR clusters.

4.4.1 Carbachol-induced AChR cluster loss on myotubes expressing WT-rapsyn-EGFP

To identify an appropriate concentration of carbachol, the effects of carbachol were tested on myotubes expressing WT-rapsyn-EGFP. The myotubes were treated with full-length human agrin (FLagrin) at a predetermined concentration, 1 in 50 dilution, for 16 hours to induce AChR clustering. Subsequently, the excess FLaGrin was washed off and replaced by either 50 μ M or 100 μ M of carbachol in the culture medium. To evaluate if agrin removal affects the stability of AChR clusters, additional conditions, agrin remained on and agrin washed off, were also included in this experiment. The cells were labelled with α -BuTx-594 and fixed at 0, 8 and 20 hours after the initial 16 hours of agrin treatment, and a total of 60 microscopic fields from 3 separate experiments were analysed for every time point of each condition. The results are presented as percentages relative to the time point 0 hours for each respective treatment condition (Figure 4-5 and 4-6).

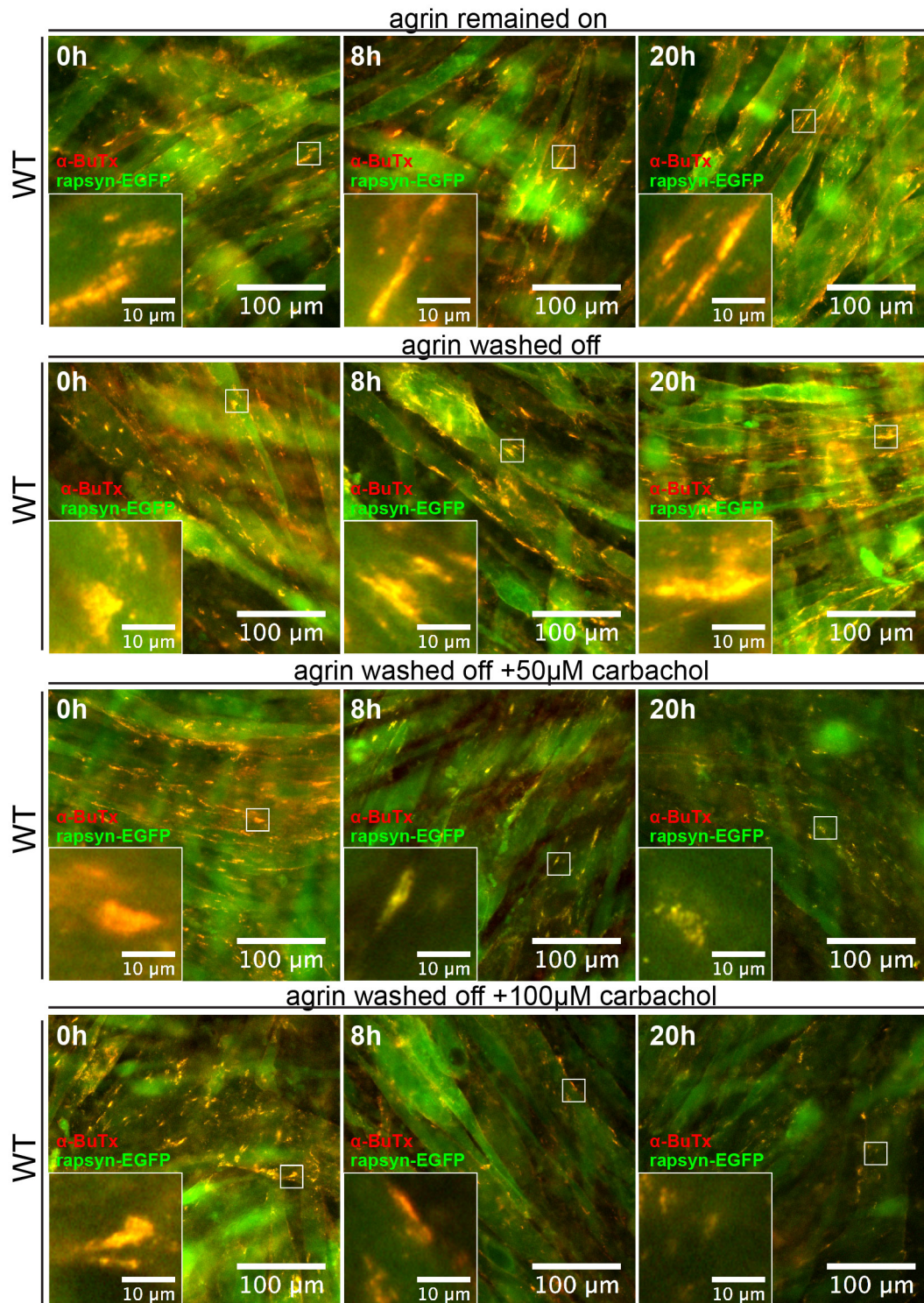


Figure 4-5 Representative images of carbachol induced AChR cluster loss on myotubes expressing WT-rapsyn-EGFP

Myotubes expressing WT-rapsyn-EGFP were treated with FLagrin for 16 hours to stimulate AChR clustering. Subsequently FLagrin was removed and replaced by 50μM or 100μM carbachol. AChR clusters were labelled with α-BuTx-594 and the cells were fixed at 0, 8 and 20h after the initial 16h of agrin treatment (images represent part of a microscopic field).

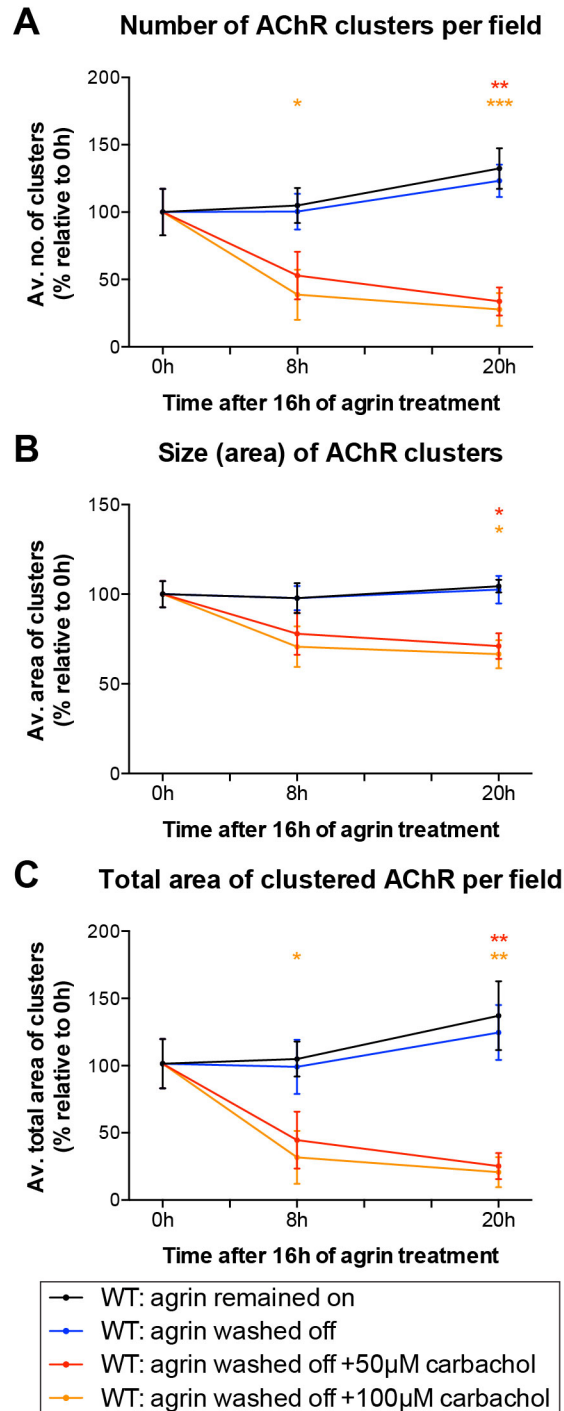


Figure 4-6 Quantified data on carbachol-induced AChR cluster loss on myotubes expressing WT-rapsyn-EGFP

Myotubes expressing WT-rapsyn-EGFP were treated with FLa-grin for 16 hours, and then excess FLa-grin was washed away and replaced with 50µM or 100µM carbachol. Myotubes were labelled with α -BuTx-594 and fixed at 0, 8 and 20 hours after the initial 16 hours of agrin. 60 images from 3 experiments were analysed and the data are normalised to 0 hours for each condition as mean % $\pm$ SEM. Two-way ANOVA with Dunnett's multiple comparisons tests, asterisks: *** p <0.001, ** p <0.01, * p <0.05

After 16 hours of FLagrin stimulation, a further 20 hours of agrin treatment slightly increased the number and total area of AChR clusters present per microscopic field, but the differences were not statistically significant. The removal of agrin did not affect the number or the total area of AChR clusters present on myotubes over time. The average size of AChR clusters remained fairly constant when FL agrin was either remained in or was removed from the culture medium.

When myotubes were exposed to 50 μ M or 100 μ M of carbachol for 20 hours, the number, average size and total area of AChR clusters per microscopic field were significantly less when compared to the agrin washed off control.

The overall effects of 50 μ M and 100 μ M carbachol on AChR clusters formed were found to be similar. 100 μ M of carbachol caused a slightly greater loss in the number and total area of AChR clusters after 8 hours of treatment, which were also detected to be statistically significant when compared to the agrin washed off control. The differences between the effects of the 2 concentrations of carbachol in the 3 parameters analysed were tested statistically, and all were found to be insignificant.

In summary, the removal of excess FLagrin alone did not induce AChR cluster loss on myotubes expressing WT-rapsyn-EGFP that were pre-treated with FLagrin for 16 hours. During the 20 hours of monitoring, the exposure of myotubes to carbachol reduced the number, average size and total area of the AChR clusters remaining on myotubes over time. As both concentrations of carbachol demonstrated similar effects towards AChR cluster loss, 50 μ M was chosen to be the concentration of carbachol used to induce AChR cluster loss on myotubes in subsequent experiments.

4.4.2 The levels of AChR on surfaces of myotubes expressing WT-rapsyn-EGFP after carbachol treatment

To understand the consequential localisation of AChR after the clusters were broken down, the ^{125}I - α -BuTx binding assay was performed to measure the levels of cell surface AChR (n=3). The ^{125}I -BuTx binding assay was performed on myotubes expressing WT-rapsyn-EGFP at 0, 6 and 24 hours after the initial 16 hours of agrin treatment, and the relative levels of ^{125}I - α -BuTx bound on myotubes surfaces were normalised to the data for 0 hours for each of the conditions (Figure 4-7).

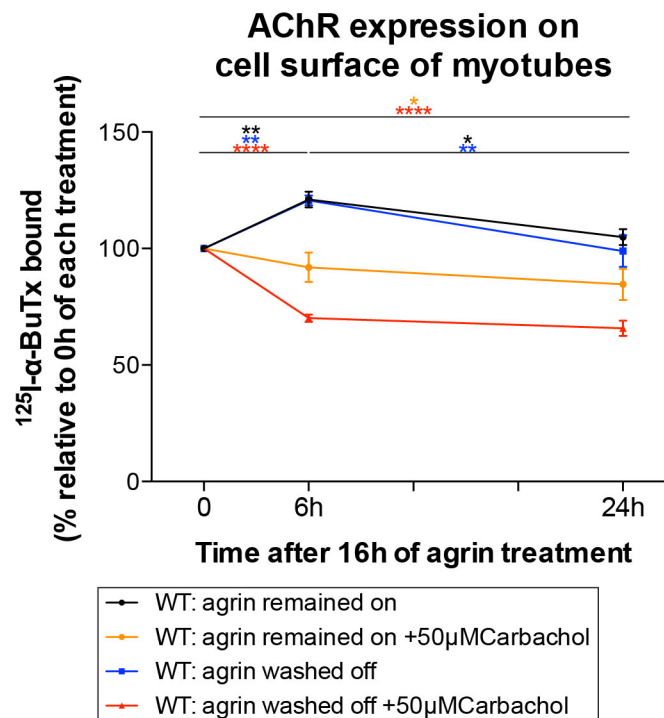


Figure 4-7 Effects of carbachol towards the expression of cell surface AChR on myotubes expressing WT-rapsyn-EGFP

Myotubes expressing WT-rapsyn-EGFP were incubated with FLagrin for 16 hours. Subsequently, excess FL were either washed off or left in the culture medium, with or without the addition of 50 μM carbachol. The expression of surface AChR were measured by ^{125}I - α -BuTx binding assay at 0, 6 and 24 hours after the initial 16 hours of FLagrin incubation. The results are normalised to time 0 for each treatment condition, and presented as mean $\pm$ SEM (n=3). Two-way ANOVA with Tukey's multiple comparisons test, asterisks: ****p<0.0001, ***p<0.001, **p<0.01 and *p<0.05.

The levels of ^{125}I - α -BuTx bound on the cell surface of myotubes that had different treatment conditions were compared. The addition of 50 μM carbachol to cells that had excess agrin left on or removed reduced the relative levels of ^{125}I - α -BuTx bound significantly at both 6 ($p=0.0007$, $p<0.0001$ respectively) and 24 hours ($p=0.0313$, $p=0.0001$ respectively). This suggests that carbachol induces the loss of AChR expression on cell surface. At 6 hours, the relative ^{125}I - α -BuTx bound on myotubes that had excess agrin and carbachol in the culture medium were higher compared to myotubes that had FLagrin removed and carbachol ($p=0.0164$). This difference was still evident at 24 hours, but the statistical p -value was marginally outside the range of significance ($p=0.0597$). Hence the data suggests that the presence of agrin may help to stabilise the expression of surface AChR.

In summary, the results indicate that the carbachol treatment reduces the expression of AChR on myotubes surfaces. This can be due to the loss of AChR clusters or because carbachol may have a direct effect on the turnover rates of surface AChR. The removal of excess agrin did not affect AChR surface expression, but in the presence of carbachol, agrin seem to have a stabilising effect on AChR expression.

4.4.3 Effects of *RAPSN* mutations on the stability of AChR clusters

The stability of AChR clusters formed on myotubes expressing V45M-, N88K-, R91L- and A153T- rapsyn-EGFP was investigated. FLaGrin was used to stimulate AChR clustering, and the excess was washed off, and 50 μ M of carbachol was applied to the cells to induce AChR cluster loss. The myotubes were labelled with α -BuTx-594 and fixed at 0, 1.5, 3, 6, 12 and 24 hours after exposure to carbachol. Example images of part of a microscopic field for the effects of 50 μ M carbachol on the AChR clusters present on myotubes expressing the rapsyn-EGFP variants at 0, 6 and 24 hours can be found in Figure 4-8. For each of the rapsyn-EGFP variants, a total of 60 microscopic fields from 3 individual experiments were analysed for every time point. The data were normalised to 0 hours for each of the variants investigated (Figures 4-8, 4-9, 4-10 and 4-11).

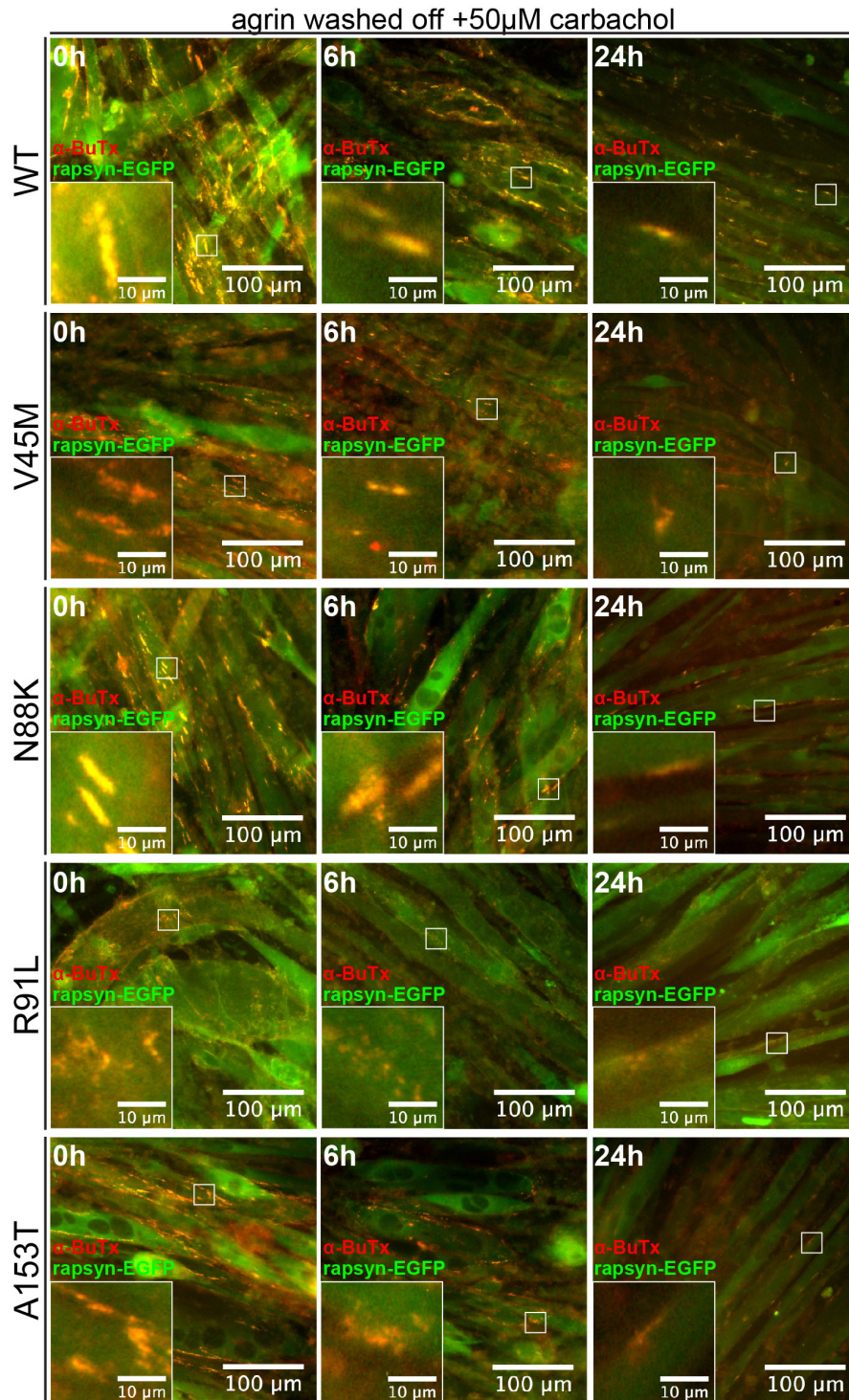


Figure 4-8 Representative images of carbachol induced AChR cluster loss on myotubes expressing WT- and mutant- rapsyn-EGFP

Myotubes expressing WT-, V45M-, N88K-, R91L- and A153T- rapsyn-EGFP were treated with FLaGrin for 16 hours to stimulate AChR clustering. Subsequently, FLaGrin was removed and replaced by 50μM carbachol in the culture medium. The myotubes were labelled with α-BuTx-594 and fixed at 0, 1.5, 3, 6, 12 and 24 hours after agrin was removed (images represent part of a microscopic field at 0, 6, and 24 hours).

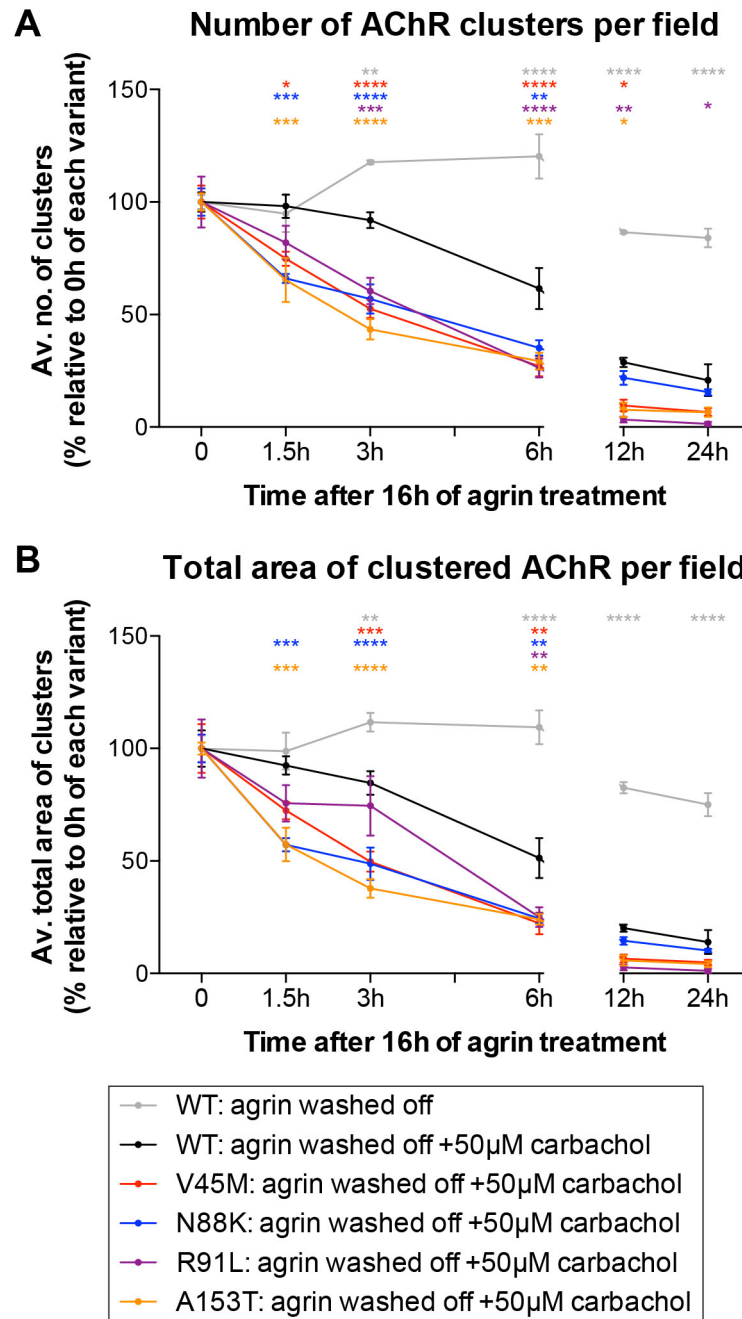


Figure 4-9 Quantified data on carbachol-induced AChR cluster loss on myotubes expressing WT- and mutant-rapsyn-EGFP

Myotubes expressing WT- or mutant-rapsyn-EGFP were treated with FLaGrin for 16 hours, and then excess FLaGrin was washed away and replaced with 50µM carbachol. Myotubes were labelled with α -BuTx-594 and fixed at 0, 1.5, 3, 6, 12 and 24 hours after the initial 16 hours of agrin stimulation. 60 images from 3 experiments were analysed and data were normalised to 0 hours for each condition as mean percentages $\pm$ SEM. Two-way ANOVA with Dunnett's multiple comparisons tests, asterisks: **** p <0.0001, *** p <0.001, ** p <0.01, * p <0.05

After 1.5 hours of carbachol treatment, the relative percentage of the number of AChR clusters per field remained fairly constant for clusters formed by WT-rapsyn-EGFP, but fewer AChR clusters remained on myotubes expressing V45M-, N88K- and A153T- rapsyn-EGFP. At 3 hours, the relative percentages of the number of AChR clusters remained on myotubes expressing the mutant-rapsyn-EGFP investigated were significantly less than myotubes expressing WT-rapsyn-EGFP. The statistical differences continued for the time points 6 and 12 hours, except for myotubes expressing N88K at 12 hours. After 24 hours of exposure to carbachol, the relative percentages of the number of AChR clusters remained on myotubes expressing the mutant rapsyn-EGFP were on average less than WT, but except for R91L-rapsyn-EGFP, the differences were no longer significant (Figure 4-9 A).

The changes in the total area of clustered AChR on myotubes surfaces upon carbachol treatment agree with the results on the number of AChR clusters that remained per microscopic field. After 1.5 hours of carbachol treatment, the relative percentages of the total area of AChR clusters per field on myotubes expressing N88K- and A153T- were found to be significantly less than myotubes expressing WT-rapsyn-EGFP. The significance in the differences was also detected at 3 and 6 hours. At 3 hours, the difference between WT and V45M became significant. After 6 hours of carbachol treatment, the relative percentages for the total area of AChR clusters remained for myotubes expressing mutant-rapsyn-EGFP were less than WT. However, from 12 hours of carbachol treatment, no statistical differences in the relative percentages of the remaining total area of AChR clusters were detected between myotubes expressing WT- and mutant- rapsyn-EGFP (Figure 4-9 B).

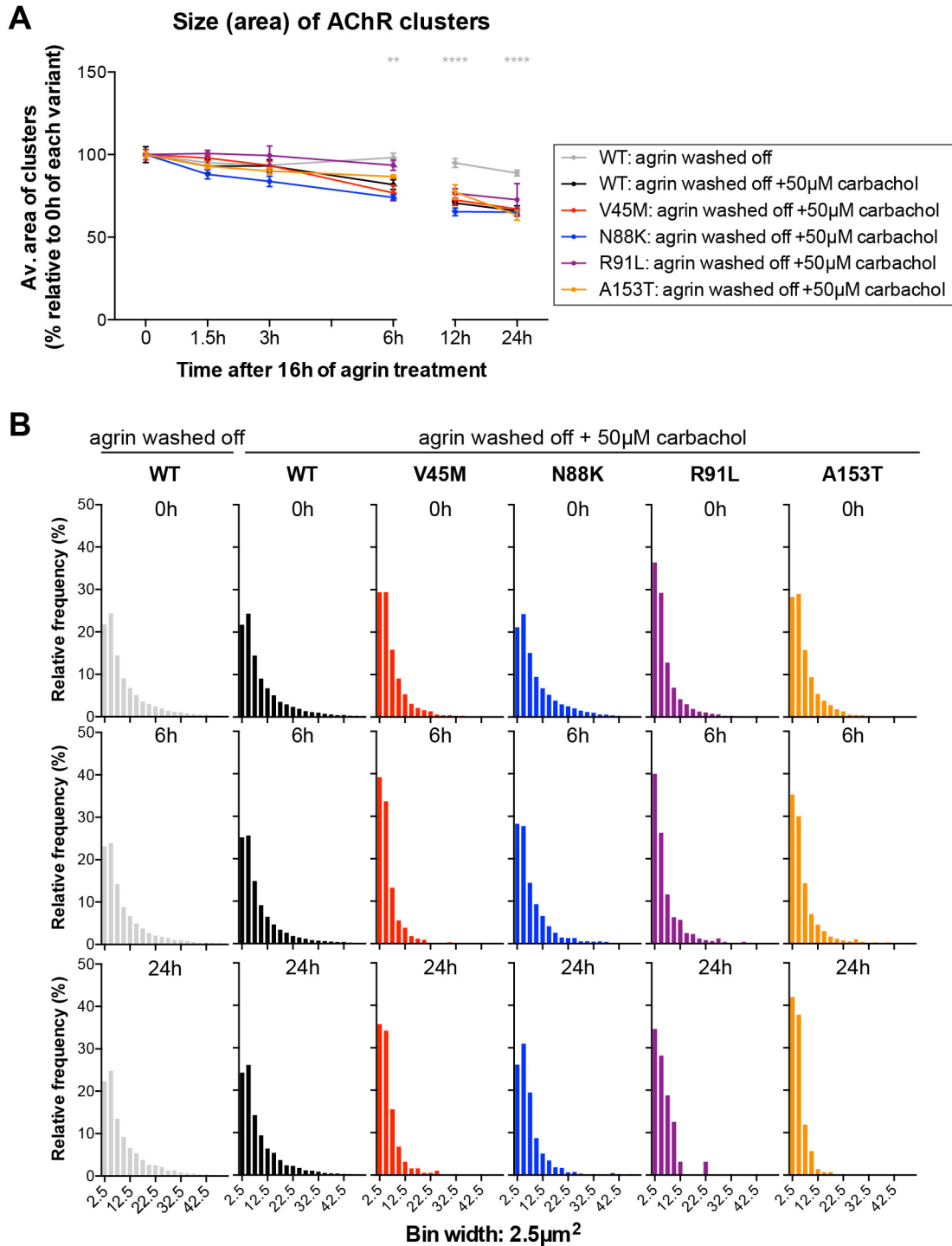


Figure 4-10 The effect of carbachol treatment on the average size of AChR clusters

(A) 60 images from 3 experiments were analysed and data were normalised to 0 hours for each condition as mean percentages $\pm$ SEM. Two-way ANOVA with Dunnett's multiple comparisons test, asterisks: **** p <0.0001, ** p <0.01. (B) AChR clusters were categorised by size and the relative frequency distributions at 0, 6 and 24 hours were plotted on histograms.

Initial analysis of the average size of AChR clusters formed per field suggested the relative changes between myotubes expressing WT- and any of the mutant-rapsyn-EGFP were similar (Figure 4-10 A). To identify subtle changes that may have occurred in the size of AChR clusters over the duration of carbachol treatment, the data collected at 0, 6 and 24 hours after agrin was washed off were plotted on a histogram to display the relative frequency of AChR clusters of different sizes in percentages. The results suggested an increased proportion of the larger AChR clusters formed by mutant-rapsyn-EGFP were lost after 24 hours of carbachol treatment (Figure 4-10 B).

To extend the analysis, the relative frequencies of large AChR clusters ($>10\mu\text{m}^2$) after 0 and 24 hours of exposure to carbachol were compared.

The relative percentages of large AChR clusters

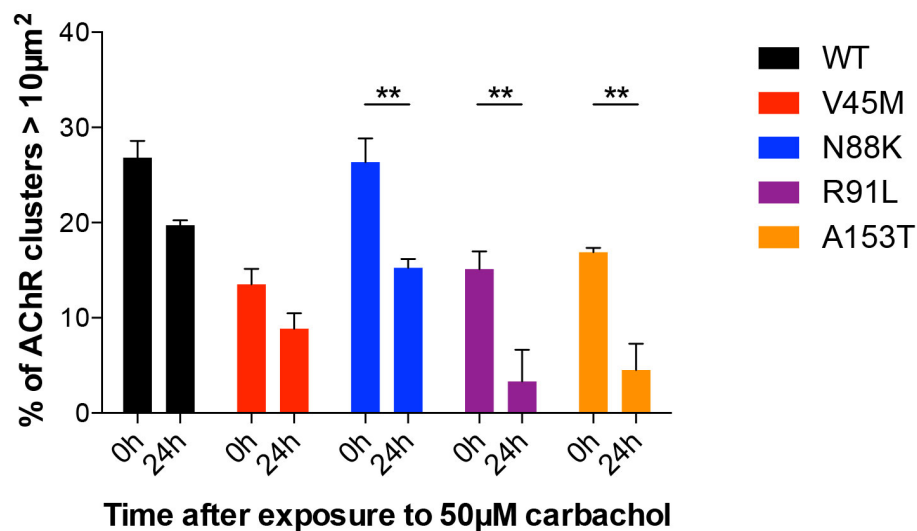


Figure 4-11 The relative frequencies of large AChR clusters that remained after 24 hours of carbachol treatment

The relative frequencies of AChR clusters larger than $10\mu\text{m}^2$ on myotubes expressing WT- or mutant-rapsyn-EGFP were analysed at 0 and 24 hours after exposure to $50\mu\text{M}$

carbachol. Each bar represents the mean $\pm$ SEM (n=3). Two-way ANOVA and Sidak's multiple comparisons tests, asterisks **p<0.01

The relative percentages of large clusters remained fairly constant for WT- and V45M-rapsyn-EGFP expressing myotubes. However, the relative percentages of large AChR clusters on myotubes expressing N88K-, R91L- and A153T-rapsyn-EGFP were significantly reduced (Figure 4-11).

Thus, the results from the number and total area of AChR clusters per field provided clear evidence that AChR clusters formed by the mutant-rapsyn-EGFP were less stable. In addition, analysis of the relative changes in the size of AChR clusters showed that carbachol also induces greater loss of larger AChR clusters on myotubes expressing N88K- R91L-, and A153T-rapsyn-EGFP.

4.4.4 Effects of *RAPSN* mutations on the internalisation of AChR after the disassembly of AChR clusters

The mutations in *RAPSN* may modulate the turnover of AChR on cell surface, and cause increased internalisation once AChR clusters have broken down. To test this hypothesis, the carbachol induced changes on the levels of cell surface AChR on myotubes expressing WT- and mutant- rapsyn-EGFP at 0, 6 and 24 hours after 16 hours of FLagrin stimulation were measured by the ^{125}I - α -BuTx binding assay (n=3). *Rapsn*-KO myotubes expressing EGFP were also included in the experiment to gain an understanding about the carbachol-induced surface AChR loss. (Figure 4-12)

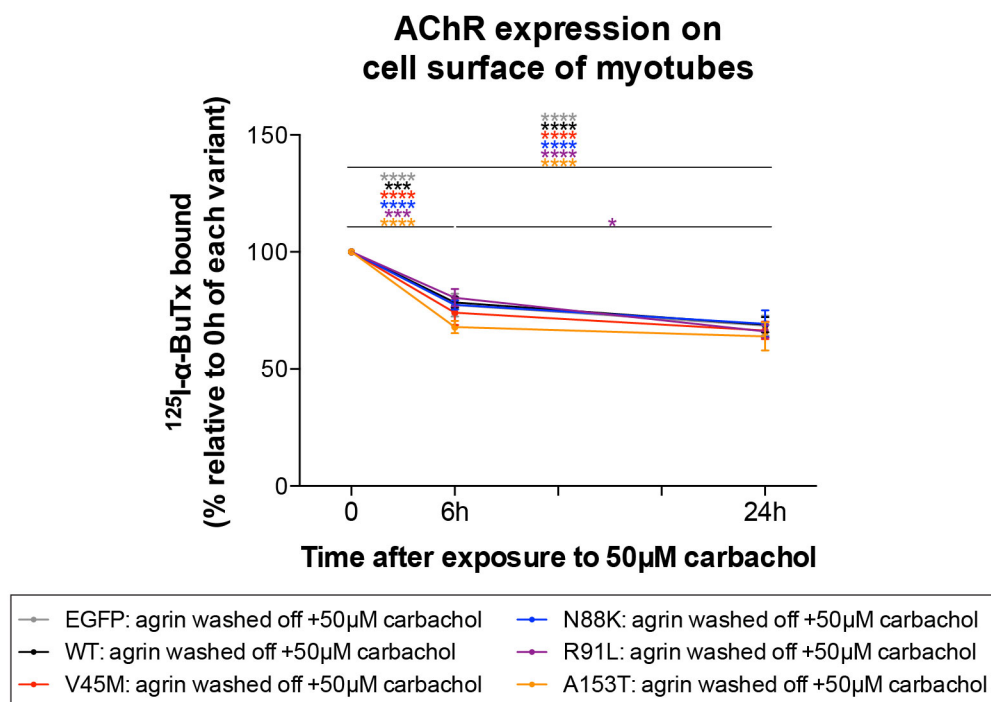


Figure 4-12 Expression of cell surface AChR on myotubes expressing rapsyn-EGFP variants after exposure to carbachol

Myotubes expressing WT- or mutant-rapsyn-EGFP, or EGFP were incubated with FLagrin for 16 hours, subsequently, excess FLagrin were removed and replaced by 50µM carbachol. The levels of cell surface AChR were measured by ^{125}I - α -BuTx binding at 0, 6 and 24 hours after exposure to carbachol. The results, mean $\pm$ SEM (n=3), are normalised to time 0 hours for each variant. Two-way ANOVA with Tukey's multiple comparisons test, asterisks: ****p<0.0001, ***p<0.001, **p<0.01 and *p<0.05.

No significant differences were detected when the levels of ^{125}I - α -BuTx bound on myotubes expressing EGFP or mutant-rapsyn-EGFP were compared with WT. Myotubes expressing A153T-rapsyn-EGFP had a slightly more reduced level of ^{125}I - α -BuTx compared to other variants after 6 hours, but when this was compared to WT, the difference was not significant ($p=0.1210$) (Figure 4-12).

The similarity in the pattern of reduced levels ^{125}I - α -BuTx bound on myotubes expressing EGFP or WT-rapsyn-EGFP suggests that carbachol has direct effects on the turnover of surface AChR that is independent to AChR cluster loss. However, the reduction in surface AChR did not correlate with the relative percentages of AChR cluster loss. Since ^{125}I - α -BuTx binds to all AChR on myotube surfaces, including both clustered and non-clustered AChR, the specific localisations of AChR after cluster disassembly cannot be confirmed by this experiment.

4.5 The effects of *RAPSN* mutations on the dynamics of AChR and rapsyn-EGFP within clusters

4.5.1 Experimental protocols

Rather than just investigating AChR clusters, I was also interested in studying the dynamics of AChR and rapsyn molecules within clusters. A possible approach that is compatible with the *Rapsn*-KO model system is fluorescence recovery after photobleaching (FRAP), but the application of FRAP analysis for studying AChR clusters in the *Rapsn*-KO system required optimisation.

Clusters of AChR and rapsyn were analysed one at a time, and the mobility of AChR labelled with α -BuTx-594 and rapsyn-EGFP within the same clusters were investigated (Figure 4-13). For each of the clusters studied, a total of 200 images were acquired continuously at 242.04 msec per frame, and the photobleaching occurred just after the 25th frame. The mean fluorescence intensities of 2 rectangular regions that overlap with the AChR clusters, at separate locations, were measured to generate FRAP data. 1) The control box: a region where no photobleaching would be applied, and the mean fluorescence intensity of this region for the duration of the FRAP experiment would indicate if imaging of the clusters has caused photobleaching. 2) The bleach box: a region where both α -BuTx-594 and rapsyn-EGFP would be photobleached by lasers specific for 594nm and 488nm respectively at 100% laser power, and the subsequent recovery of fluorescence intensity within this region is monitored.

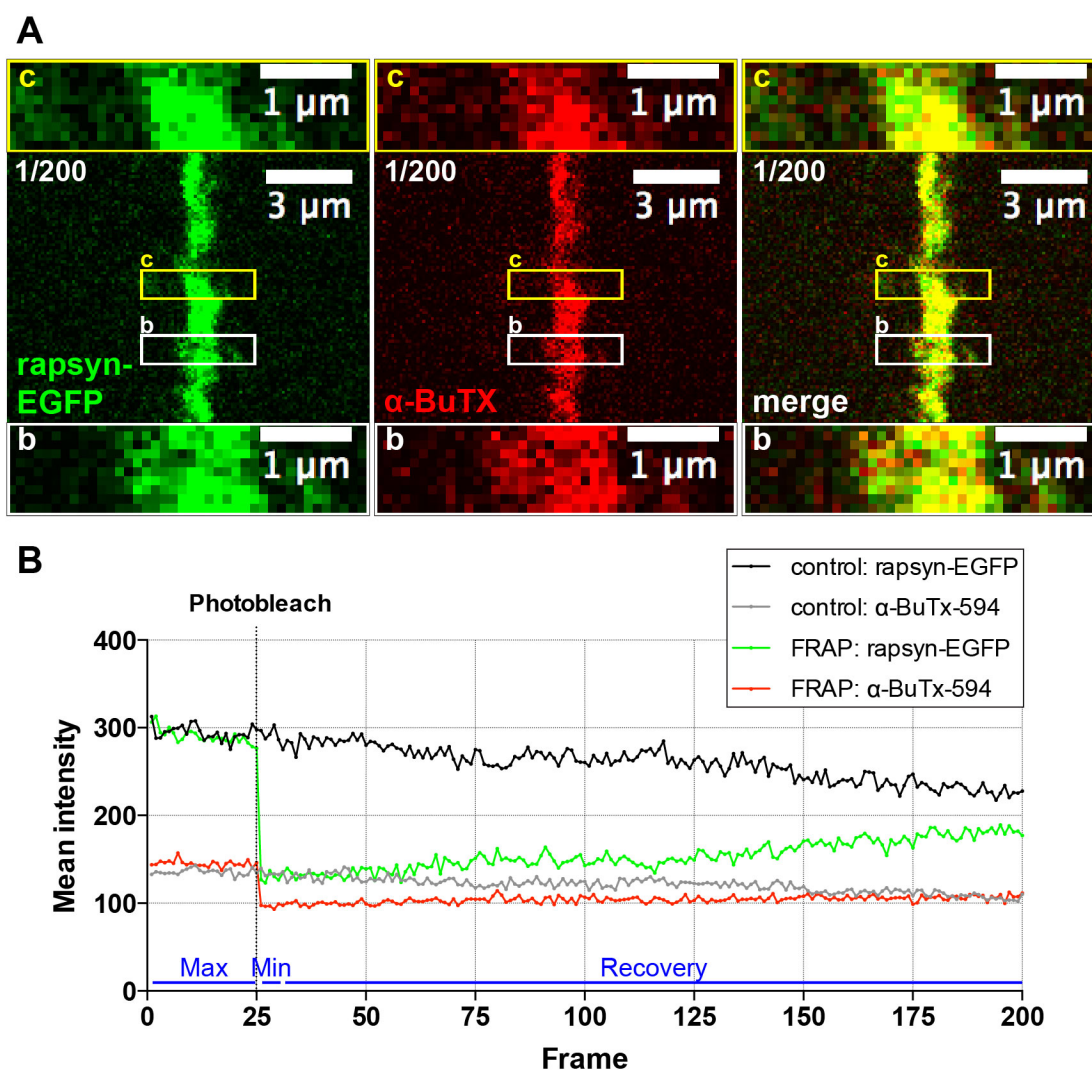


Figure 4-13 The basic design of FRAP analysis for AChR clusters

- (A)** Clusters of rapsyn-AChR were analysed individually. EGFP-tag on rapsyn and the labelling of AChR with α -BuTx-594 allows the lateral mobility of these proteins to be studied. 200 images were acquired continuously for each cluster, and the images represent the first of the 200. Rectangular regions that overlap with the clusters were used to collect data on the mean fluorescence intensities. The yellow box-c, the control box, is the region where no photobleaching would be applied. The white box-b, the bleach box, is the region where both rapsyn-EGFP and α -BuTx-594 would be photobleached by a high intensity laser after the 25th frame of the imaging sequence.
- (B)** The mean intensity of both the control and bleach box regions measured from the 200 images of the cluster shown in A confirms photobleaching and fluorescence recovery. However, the reduction in mean intensity for control over time suggests that the method of analysis needed to be optimised.

Thus the mean fluorescence intensities of the bleach box from the initial 25 frames of the experiments would indicate the original (maximum) intensity of the cluster. Immediately after photobleaching, the mean fluorescence intensity of the bleach box would represent the minimum intensity, and the difference between the maximum and minimum intensity dictates the relative depth of photobleaching. Images taken after photobleaching allow the changes in the fluorescent intensities, within the predefined regions, during the recovery phase of the experiment to be monitored. Hence an increase in fluorescence intensity in the bleached region over time would dictate the mobility of the fluorescently labeled/tagged proteins.

When the mean intensities of the control and bleach boxes of the example cluster were plotted on a graph (Figure 4-13 **B**), reductions in the mean intensity after photobleaching were evident for both rapsyn-EGFP and α -BuTx-594. Fluorescence recovery was also detected for both channels.

The data highlighted 2 issues in the experimental design that needed to be addressed. The maximum intensity and the bleach depth for rapsyn-EGFP and α -BuTx-594 differed by over two-fold. This likely stems from the differences of rapsyn-EGFP being a fluorescent protein and AChR being labelled by α -BuTx-594. This can be overcome by normalising the recovery in fluorescence intensity to the bleach depth for each cluster investigated. Another problem was the reduction in intensity in the no-bleach control regions over time. It was identified that the localisation of the cluster had shifted within the image frame, which caused misalignments between the intended and actual regions used for measurements. This can cause misleading results for both the control and FRAP data.

The cluster was found to have shifted downwards within the image frame (Figure 4-14 **A**). By estimation, corrected control and bleach boxes (blue and red boxes respectively) were used to measure the mean fluorescence intensities of rapsyn-EGFP in the final frame of the 200 images taken for this cluster. The misaligned boxes had mean intensities of 227.835 and 177.075 for the control and bleached regions respectively, while the corrected boxes had mean intensities of 264.590 and 145.518. Thus, the detected reduction in fluorescence intensity in the control region was not due to photobleaching, but was primarily caused by movements of myotubes instead.

As it would be technically difficult to manually track the movements of AChR clusters to reposition measurement boxes for every frame acquired for each cluster with consistency, a larger box was used to measure fluorescence intensity instead. The control and bleach boxes are $4\mu\text{m} \times 1\mu\text{m}$ in size. Demonstrated by the example cluster, it only occupied the central part of the bleach box with extra spaces on each side along its x-axis. Hence if the cluster was to move horizontally, as long as the cluster is kept within the region of the bleach box, the additional space could act as a buffering system to minimise the impact of movements towards the overall mean intensity measured. To provide similar buffering spaces for the y-dimension of the cluster, a measurement box $4\mu\text{m} \times 2\mu\text{m}$ in size, providing an extra $0.5\mu\text{m}$ of space on each side of the bleach box, can be used to measure the mean intensity instead. For the example cluster, this extra space was sufficient to include the bleached region of the cluster within range for measurement (Figure 4-14 **B**). For consistency, the control box was also adapted to the size of $4\mu\text{m} \times 2\mu\text{m}$ (Figure 4-14 **C**).

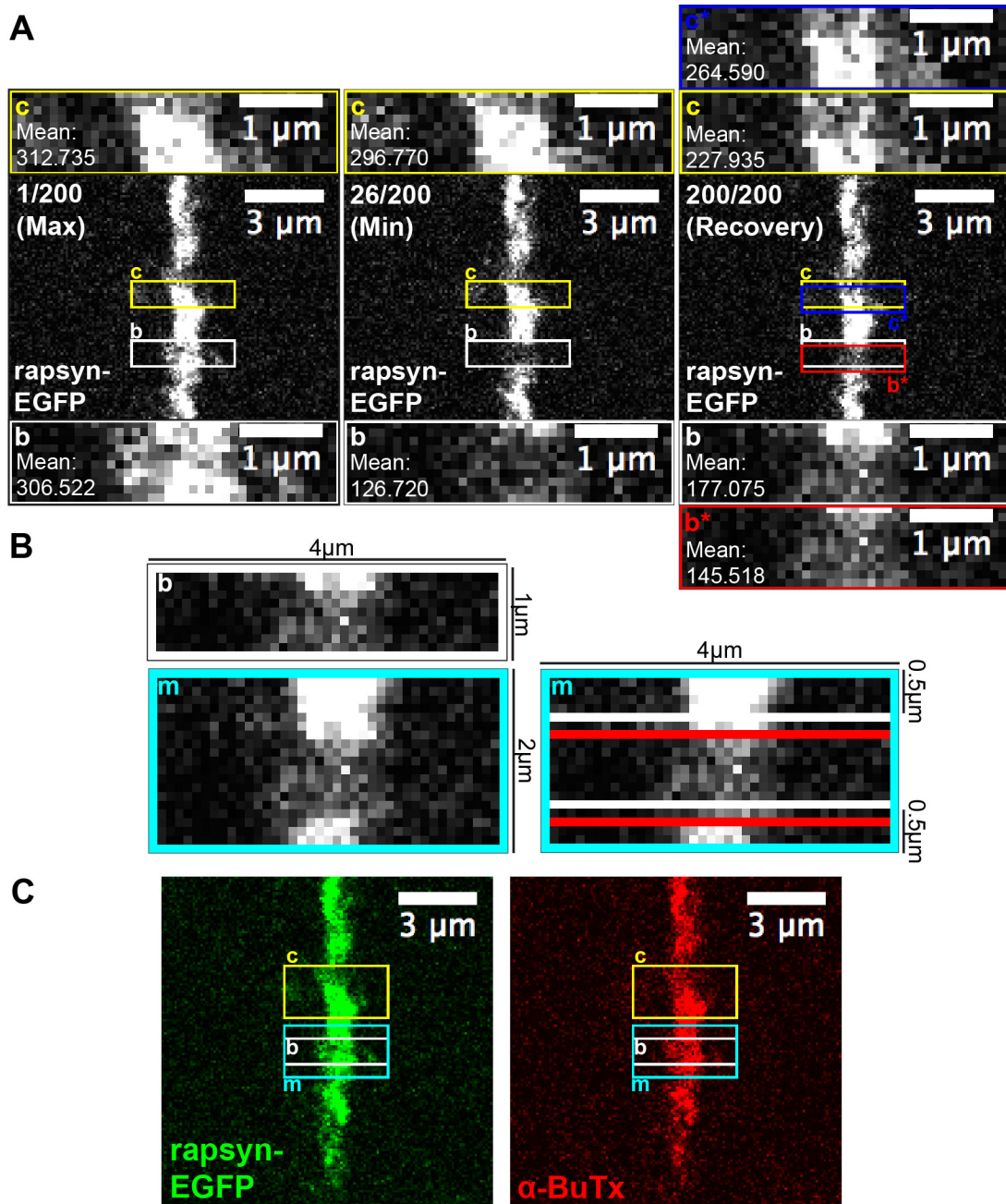


Figure 4-14 Optimisation on the analysis of FRAP data

- (A) The final frame (200/200) of the imaging series shows that the cluster had shifted down within the image frame and the measurement of the mean fluorescent intensities from the corrected control (c*-blue) and bleach boxes (b*-red) suggested that the shift could lead to misleading results.
- (B) The measurement box (m-cyan) provides extra spaces on the y-dimension and kept the bleached region in range for measuring the mean fluorescent intensities
- (C) The optimised protocol for FRAP analysis on clusters of rapsyn-EGFP and AChR. Control box (c-yellow), bleach box (b-white) and measurement boxes (m-cyan) were used to generate FRAP data.

When the images for the example cluster were reanalysed using the revised measurement settings, the no bleach controls were demonstrated to have minimal reduction over the duration of 200 frames, and the fluorescence recovery could still be detected (Figure 4-15). Therefore the optimised approach was used in subsequent FRAP analysis to investigate the effects of *RAPSN* mutations on the dynamics of rapsyn-EGFP and AChR within clusters. As a precaution, all of the image sets acquired for each cluster were evaluated, and clusters that had the bleached region moved out of the measurement box were removed from the data set to ensure the reliability of the results generated.

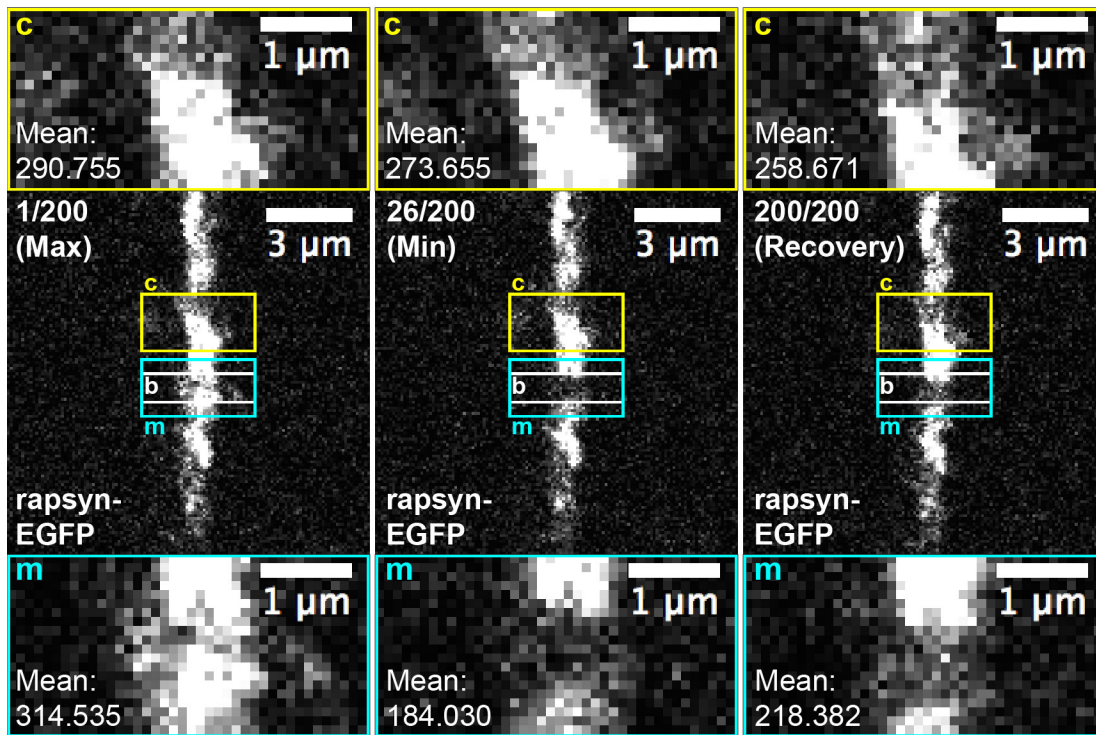
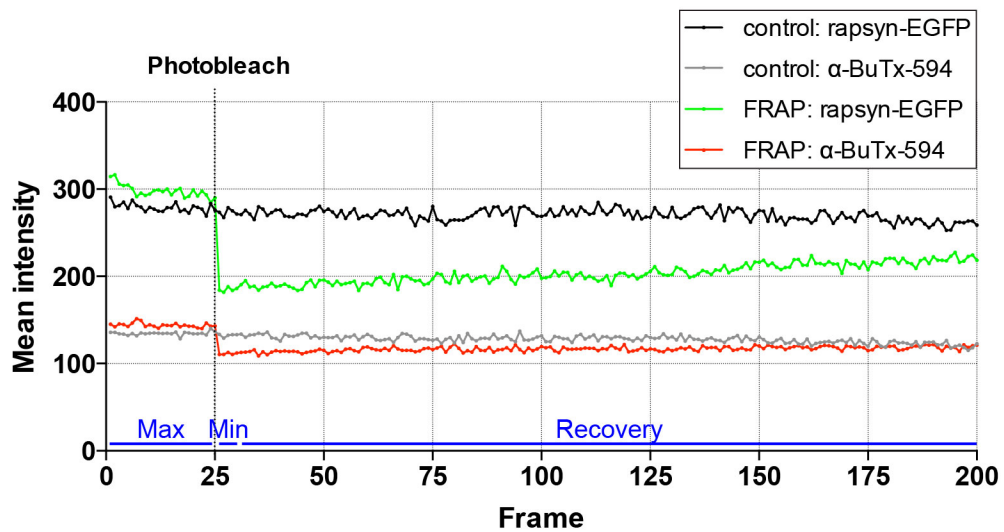
A**B**

Figure 4-15 The established FRAP analysis for AChR and rapsyn-EGFP clusters

(A) The optimised approach to measure the mean fluorescence intensities of AChR clusters was used to reanalyse the images acquired for the example cluster. Images represent the 1st (Maximum intensity), 26th (Minimum intensity) and 200th (Recovery) stages for rapsyn-EGFP.

(B) When the quantified mean intensities were plotted on the graph, the control regions of the clusters for both rapsyn-EGFP and α -BuTx-594 remained fairly constant and a steady recovery is detected from the measurement box after photobleaching of the bleach box region

4.5.2 Analysis of the mobility of rapsyn-EGFP variants within AChR clusters

Rapsn-KO myotubes expressing WT- or mutant rapsyn-EGFP were incubated with FLagrin for 16 hours to stimulate AChR clustering. Subsequently, the excess FLagrin was washed off and the AChR clusters formed on myotubes were labelled with α -BuTx-594, excess α -BuTx-594 was washed off and then the cells were subjected to FRAP analysis on an inverted confocal microscope (Zeiss LSM 780).

Due to difficulties in finding a sufficient number of clusters formed by R91L-rapsyn-EGFP that are suited for the analysis, the effect of R91L could not be investigated in this experiment. For each of the remaining rapsyn-EGFP variants, imaging series (200 frames) for 60 clusters from 3 experiments were acquired. Clusters that had the bleached region shifted out of the measurement boxes were eliminated, but no less than 50 clusters were included for each rapsyn-EGFP variant. Example images of the no bleach controls are shown in Figure 4-16, and the bleached regions in Figure 4-17.

The relative fluorescence recovery was normalised to the bleach depth of each of the clusters analysed. Mean fluorescence intensity from the region of frames 1-5 was used to represent maximum intensity, 26-30 for minimum intensity, and 195-200 for the recovery achieved by the end of the experiment (Figure 4-18).

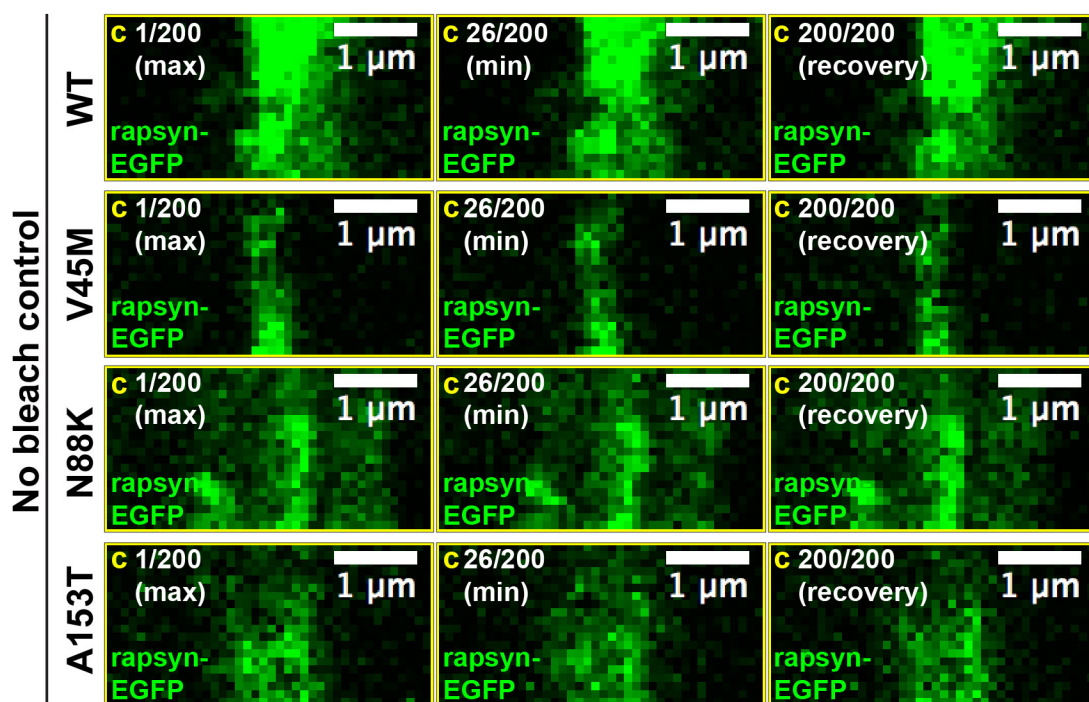


Figure 4-16 Representative images for no bleach controls of variants of rapsyn-EGFP clusters

The no bleach control regions of rapsyn-EGFP clusters formed by WT-, V45M-, N88K, and A153T- rapsyn-EGFP from the 1st, 26th and 200th image of each imaging series represent the maximum intensity, minimum intensity and recovery of rapsyn-EGFP stages of the FRAP experiment respectively.

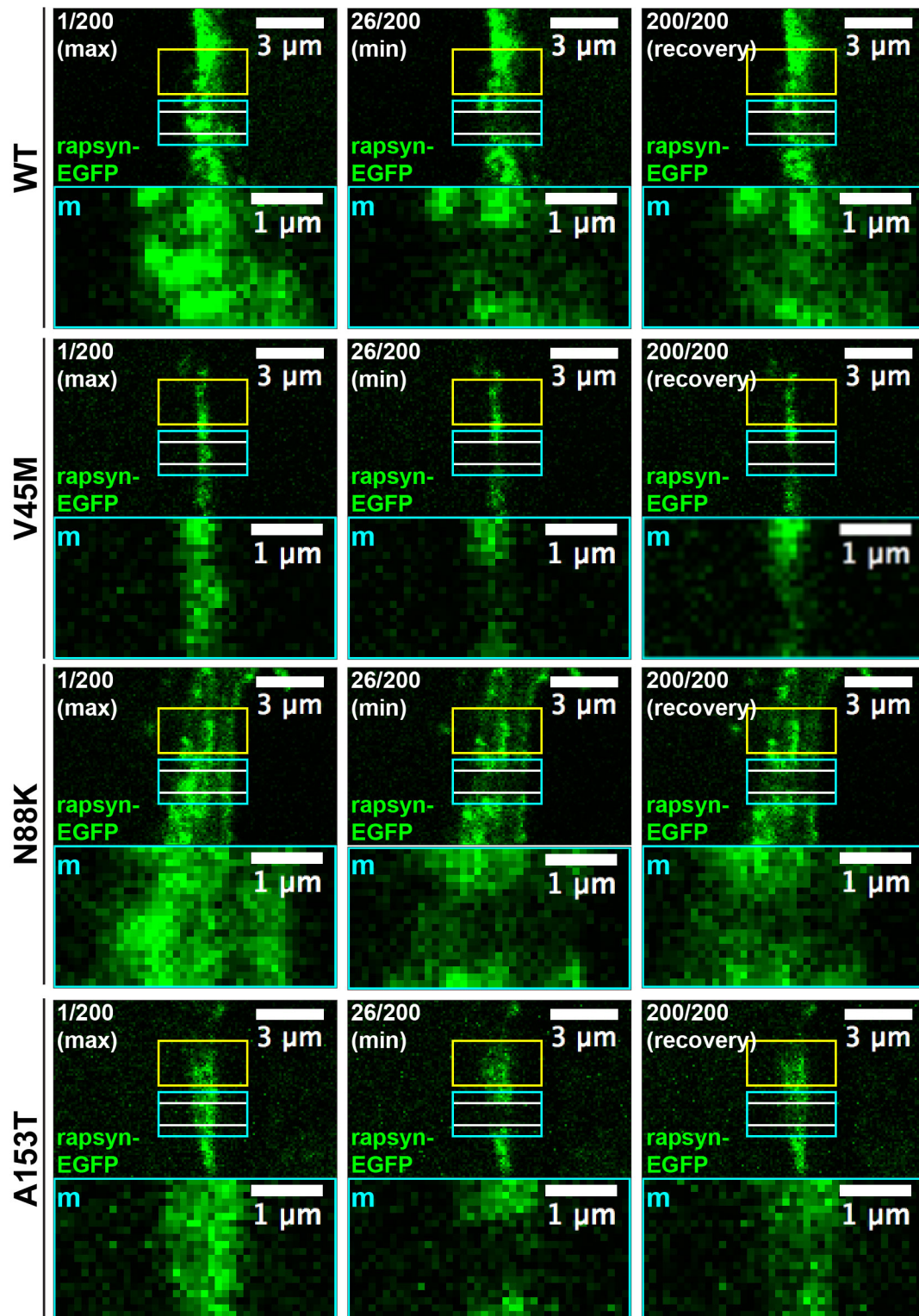


Figure 4-17 Representative images for FRAP of rapsyn-EGFP clusters

The measurement regions (m), which include the bleached region, of rapsyn-EGFP clusters formed by WT-, V45M-, N88K-, and A153T-rapsyn-EGFP from the 1st, 26th and 200th (final) image of each imaging series represent the maximum intensity, minimum intensity and recovery of fluorescence stages of the FRAP experiment respectively.

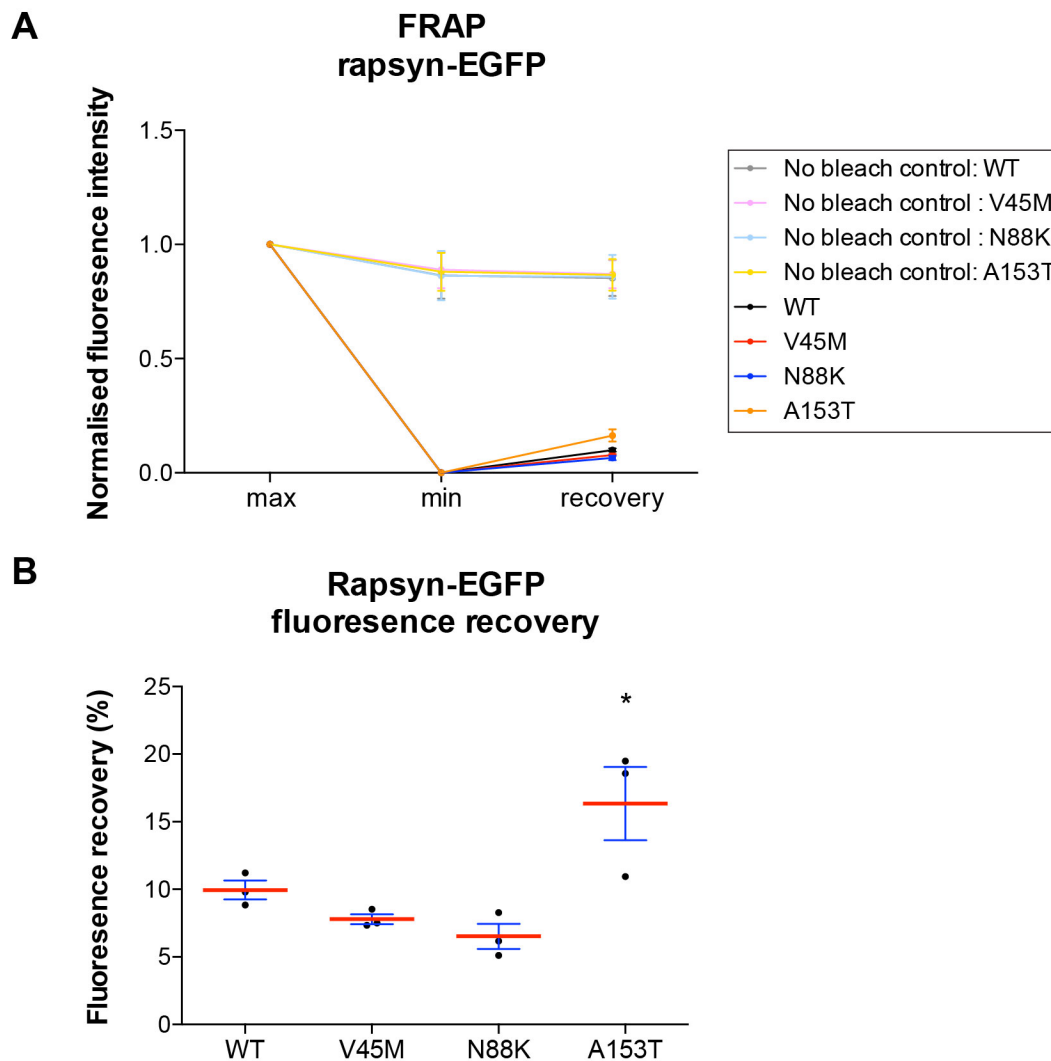


Figure 4-18 Relative fluorescence recoveries after photobleaching for variants of rapsyn-EGFP clusters

Myotubes expressing rapsyn-EGFP variants were incubated with FLAgrin for 16 hours to induce AChR clustering, and the AChR clusters formed were labelled with α -BuTx-594. The sample preparation was subjected to FRAP analysis on rapsyn-EGFP.

FRAP analysis on over 50 clusters for each of the rapsyn-EGFP variants investigated is summarised by the mean fluorescence recovery $\pm$ SEM relative to the bleach depth of each individual cluster studied. (A) The no bleach control confirms minimal non-specific photobleaching, and the relative fluorescence recovery after photobleaching in the observed period (170 continuous frames) was small. (B) The relative fluorescence recovery of V45M-, N88K- and A153T- rapsyn-EGFP was compared with WT-rapsyn-EGFP. One-way ANOVA with Dunnett's multiple comparisons test, asterisks * $p < 0.05$.

The results show that the imaging of clusters did not cause significant photobleaching to rapsyn-EGFP clusters (less than 10% for all clusters investigated), and in the duration of which the clusters were imaged for, the fluorescence recovery is very small, suggesting slow recovery that stems from the low mobility of rapsyn-EGFP within the clustered regions (Figure 4-18 **A**).

The percentage of fluorescence recovery on clusters formed with V45M- and N88K-rapsyn-EGFP was similar to WT. Significantly higher fluorescence recovery was detected on clusters formed with A153T-rapsyn-EGFP when compared to WT (Figure 4-18 **B**).

As A153T-rapsyn EGFP was able to repopulate the bleached region, the results suggest that A153T-rapsyn-EGFP is more mobile within cluster structures.

4.5.3 Analysis of the mobility of AChR within clusters formed by rapsyn-EGFP variants

The FRAP analysis for α -BuTx-594 labelled AChR clusters followed the same protocol as the investigations performed on rapsyn-EGFP, except for the laser used to introduce photobleaching, because the same clusters were analysed for both rapsyn-EGFP and α -BuTx-594 labelled AChR. Representative images of the no bleach control and bleached regions of the imaging series for each of the variants investigated can be found in Figure 4-19 and Figure 4-20 respectively. The quantified results are shown in Figure 4-21.

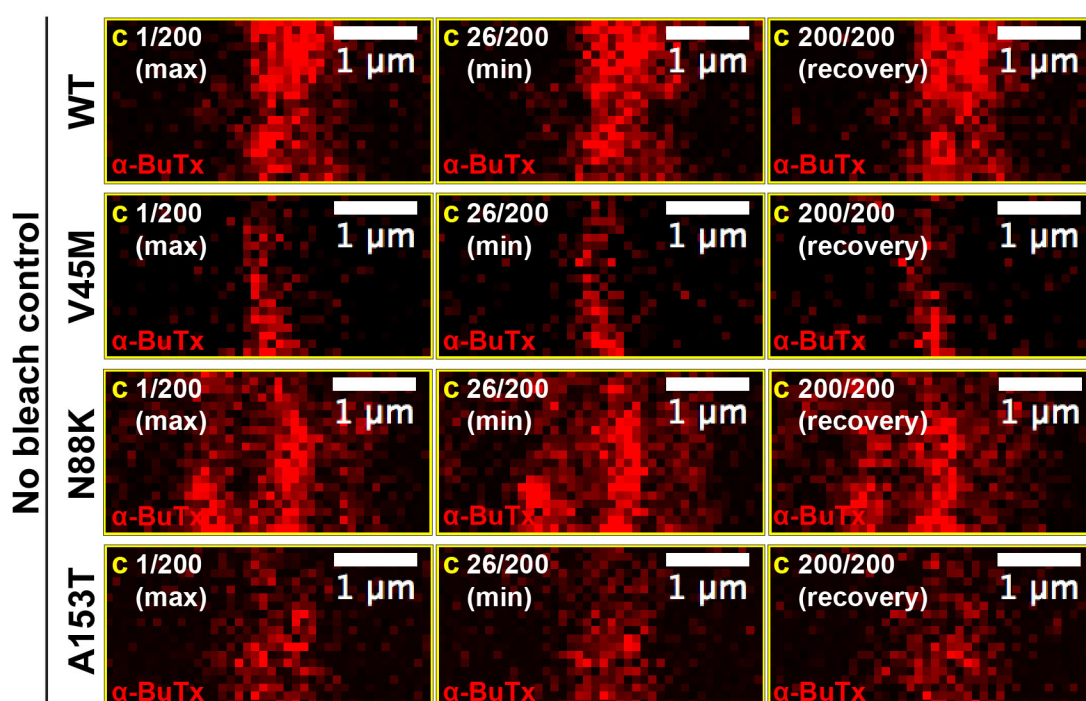


Figure 4-19 Representative images for no bleach controls of α -BuTX-594-labelled AChR clusters formed by variants of rapsyn-EGFP

The no bleach control regions of α -BuTX-594-labelled AChR clusters formed by WT-, V45M-, N88K, and A153T- rapsyn-EGFP from the 1st, 26th and 200th image of each imaging series represent the maximum intensity, minimum intensity and recovery of rapsyn-EGFP stages of the FRAP experiment respectively.

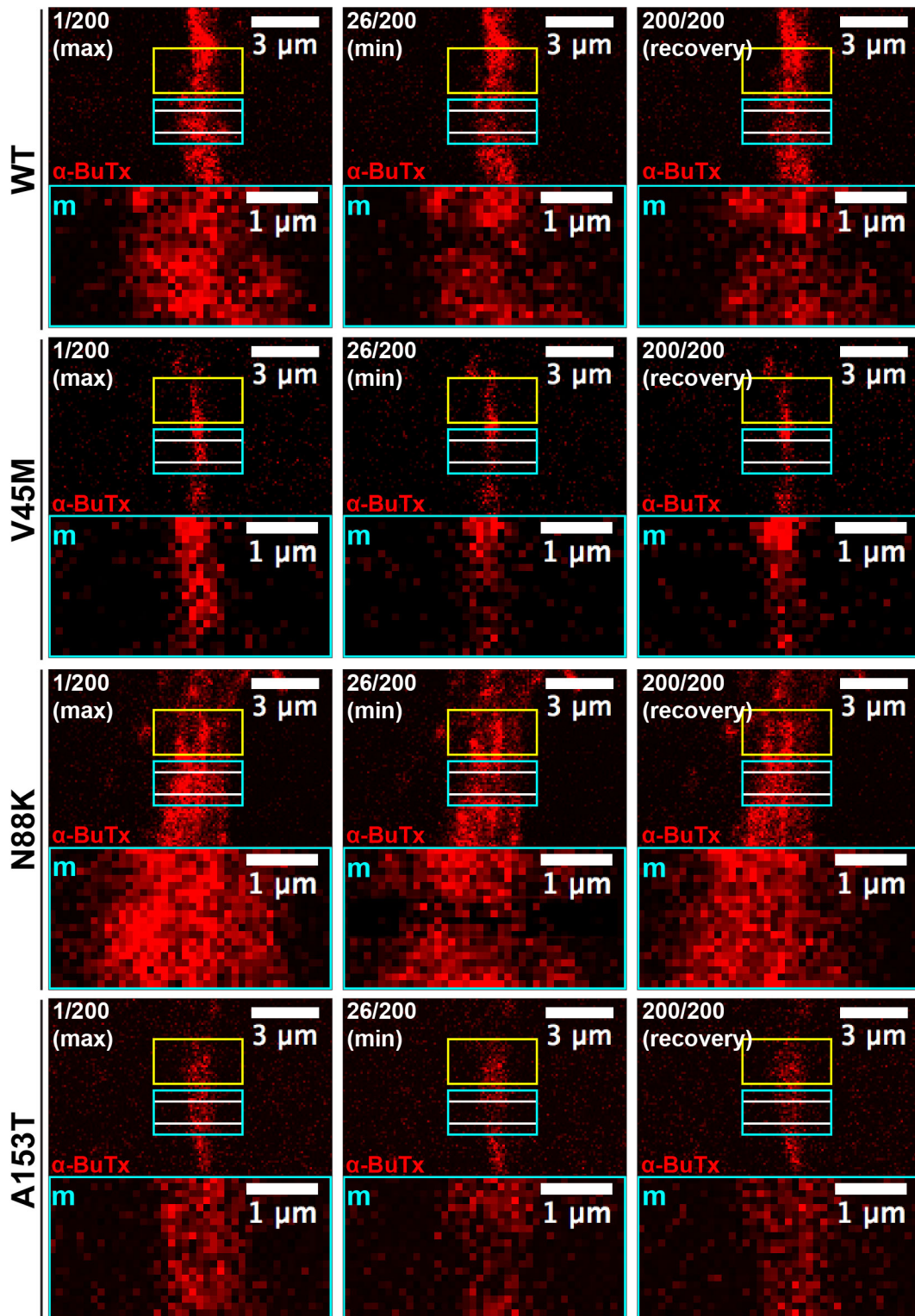


Figure 4-20 Representative images for FRAP of α -BuTX-594-labelled AChR clusters formed by variants of rapsyn-EGFP

The measurement regions (m), which include the bleached region, of α -BuTX-594 labelled AChR clusters formed by WT-, V45M-, N88K-, and A153T-rapsyn-EGFP from the 1st, 26th and 200th (final) image of each imaging series represent the maximum, minimum intensity and recovery stages of the FRAP experiment respectively.

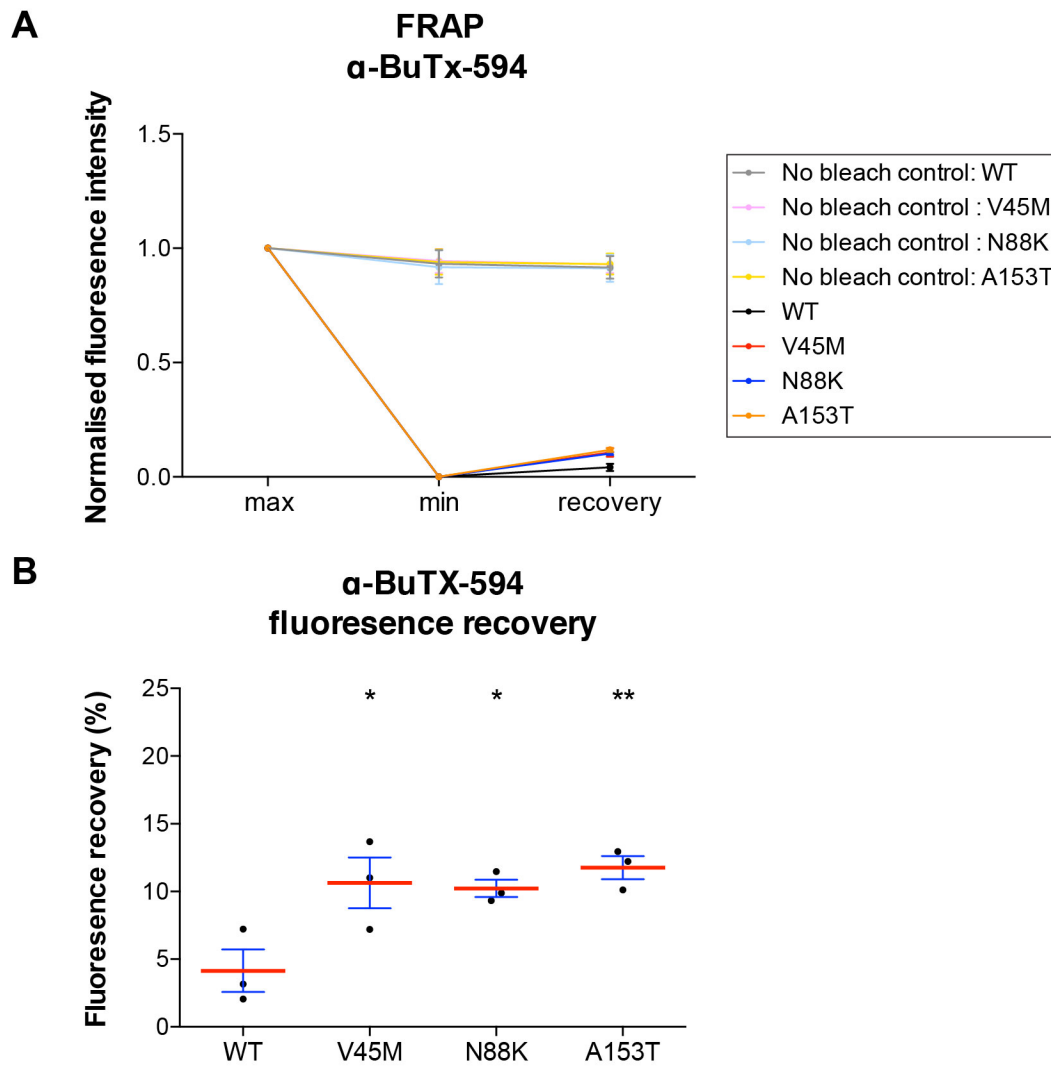


Figure 4-21 Relative fluorescence recoveries after photobleaching for AChR clusters formed by rapsyn-EGFP variants

Myotubes expressing WT- or mutant-rapsyn-EGFP were incubated with FLAgrin for 16 hours to induce AChR clustering. The AChR clusters formed were labelled with α -BuTx-594 and were subjected to FRAP analysis.

FRAP analysis on over 50 AChR clusters formed by each of the rapsyn-EGFP variants from 3 independent experiments is summarised by the mean fluorescence recovery $\pm$ SEM relative to the bleach depth of each individual cluster studied. (A) The no bleach control confirms minimal photobleaching from image acquisition, and the relative fluorescence recovery after photobleaching in the observed period was small. (B) The relative fluorescence recovery of α -BuTx-594 labelled AChR on AChR clusters formed by V45M-, N88K- and A153T- rapsyn-EGFP was compared against AChR clusters formed by WT-rapsyn-EGFP. One-way ANOVA with Dunnett's multiple comparisons test, asterisks **= $p < 0.01$, * $p < 0.05$.

Minimum photobleaching from imaging was detected, with less than a 10% reduction in fluorescence detected at the control region for each variant investigated. Similar to the results on rapsyn-EGFP, the amount of fluorescence recovery that occurred in the imaging series was small (Figure 4-21 **A**).

The relative fluorescence recoveries of α -BuTx-594 labelled AChR on AChR clusters formed by V45M-, N88K- and A153T- rapsyn-EGFP were significantly higher than clusters that were formed by WT-rapsyn-EGFP (Figure 4-21 **B**). This suggests AChR are less tethered within cluster structures that are formed by the mutant-rapsyn-EGFP investigated, which may have implications for the overall stability of AChR clusters.

Although the effects of the mutations V45M, N88K and A153T have been shown to affect the mobility of rapsyn-EGFP and/or AChR, the results need to be treated with caution due to the novelty of using FRAP to study AChR clusters in our laboratory. More detailed analyses such as the application of carbachol to AChR clusters prior to FRAP analysis with longer observation periods may further confirm the results. However, due to time limitations, I was unable to conduct further experiments.

4.6 The potential use of stimulated emission depletion microscopy to investigate protein dynamics within AChR clusters

Further experiments combining fluorescence cross-correlation spectroscopy (FCCS) with the stimulated emission depletion microscopy (STED) would be desirable. FCCS allows dual-color analysis of the fluorescence fluctuations induced by labelled particles in a confocal setup to provide information on their concentration, mobilities, molecular interactions as well as dynamic colocalisation (Bacia et al., 2006). STED microscopy can achieve spatial resolution of less than 50nm, which provides a much higher definition than traditional confocal microscopy (200nm). It employs 2 laser beams: excitation and STED depletion beams, to inhibit the fluorescence process in the outer region of the excitation point-spread function to achieve stimulated emission depletion (Hell and Wichmann, 1994). Hence an optimised setup of STED-FCCS would provide a powerful tool to study the interactive dynamics of AChR and rapsyn. Figure 21 shows an AChR cluster formed on a C2C12 myotube imaged by traditional confocal and STED microscopy (Figure 4-22).

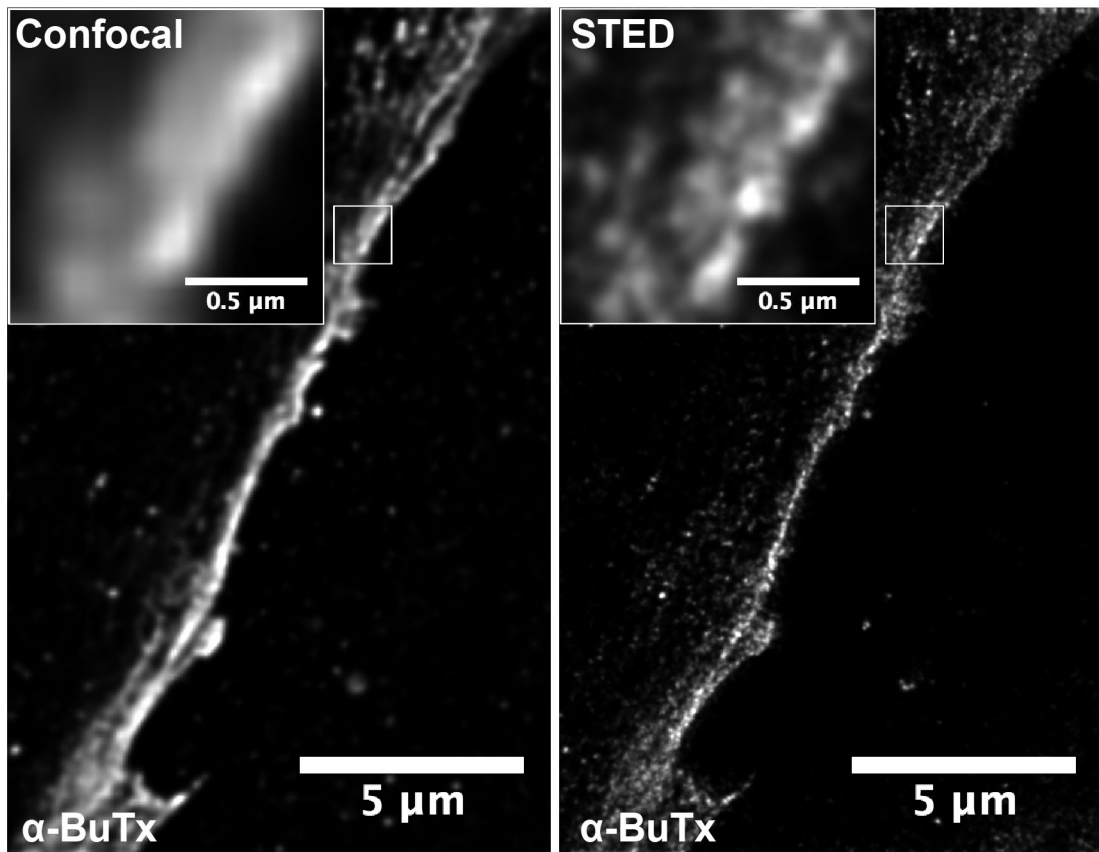


Figure 4-22 Confocal and STED images of an AChR cluster on C2C12 myotube

Full-length agrin at 1 in 100 concentration was applied to C2C12 myotubes for 16h to stimulate AChR clustering. The myotubes were then incubated with α -BuTx-488 to label AChR clusters.

The images were taken by a Leica SP8 gated STED microscope at 100x magnification. The confocal image was taken without the activation of a STED laser; and the STED image was taken with an activated STED592 laser at 75% laser power

One limiting factor is that EGFP is not optimal for STED-FCCS analysis. A second is that rapsyn is inaccessible to extracellular labelling methods. Possible solutions for this include the SNAP- and Halo- tag technologies, systems that allow fluorescent ligands that are able to cross cell membrane to bind covalently into their specific active sites. An advantage of using such systems over EGFP is the flexibility to use fluorescent ligands with different excitation/ emission wavelengths so that the combination of fluorescent dye and STED depletion laser can be tested and chosen for optimal STED microscopy.

Molecular cloning was performed to replace the EGFP-tag on rapsyn with the SNAP- or Halo- tag at the C-terminus of *RAPSN* in the pBabe-rapsyn-EGFP-PURO vector. The rapsyn fusion proteins were introduced to express in *Rapsn*-KO cells by retroviral infections, and agrin-induced AChR clustering was evaluated. On myotubes expressing WT-rapsyn- SNAP or Halo, the substrates for both tags were able to bind to their respective active sites and were colocalised with AChR clusters. However, the appearances of the AChR clusters formed were very different from the AChR clusters formed by WT-rapsyn-EGFP (Figure 4-23).

AChR clusters formed by WT-rapsyn-SNAP were present in high numbers but with small punctate morphology; on the other hand, very few AChR clusters were formed by WT-rapsyn-Halo. The effect of agrin has been tested by agrin titration experiments, and the aberrant AChR clusters formed were not dependent on the concentration of FLagrin. The protein tags may have affected the correct folding of the rapsyn protein, which is important for AChR clustering. Thus these systems for labelling rapsyn were not pursued.

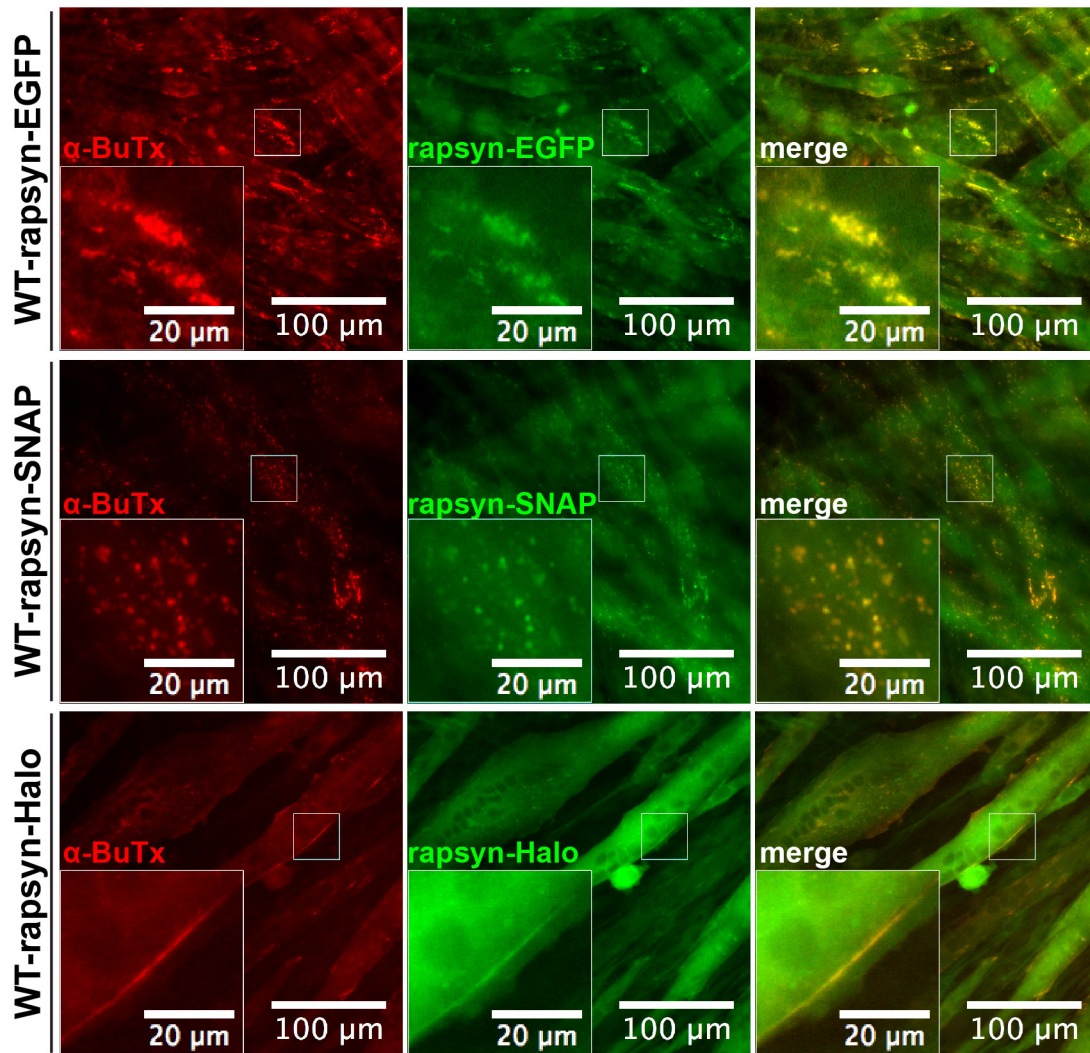


Figure 4-23 AChR clustering by WT-rapsyn- EGFP, SNAP and Halo in Rapsn-KO myotubes

Myotubes expressing WT-rapsyn- EGFP, SNAP or Halo were stimulated with FLagrin (1 in 10 concentration) for 16 hours to induce AChR clustering. To label rapsyn-SNAP and rapsyn-Halo, myotubes were incubated with SNAP-505* for 30 minutes and HaloTag ligand-TMR for 15 minutes respectively under culture conditions. Subsequently, the excess ligands were removed from myotubes and then AChR clusters were labelled with α -BuTx-594 or α -BuTx-488 respectively and fixed with 3% paraformaldehyde. For myotubes expressing WT-rapsyn-EGFP, AChR clusters were labelled with α -BuTx-594.

Each image represents part of a microscopic field.

4.7 Discussion

The pathogenic mechanisms of *RAPSN* mutations, V45M, N88K, R91L and A153T were investigated. The mutations were identified to cause multiple defects in rapsyn functioning, all of which can have implications for the stability of AChR clusters (summarised in Table 4-1). The presented findings support the hypothesis that the severe phenotypes with episodic crises in the early years of patients are associated with *RAPSN* mutations that reduce the stability of AChR clusters. The use of FRAP to study protein dynamics within AChR clusters may, in the future, allow for a better understanding of the pathogenic effects of *RAPSN* mutations on the interactions between rapsyn, AChR and other proteins that are expressed at the NMJ.

Table 4-1 Summary on the effects of *RAPSN* mutations

	V45M	N88K	R91L	A153T
Steady-state overexpression levels of rapsyn-EGFP	severely reduced	reduced	=	=
Stability of rapsyn-EGFP	severely reduced	reduced	reduced	reduced
Expression of AChR on surfaces of myotubes	reduced	reduced	=	reduced
Carbachol induced AChR cluster disassembly	reduced	reduced	reduced	reduced
Surface expression of AChR after cluster disassembly	=*	=*	=*	=*
Mobility of rapsyn-EGFP within clusters	=	=	n/a	increased
Mobility of AChR within clusters	increased	increased	n/a	increased

The pathogenic effects (red) were identified based upon comparison with WT

= = not significantly different from WT; n/a = not investigated

=* = not different from WT, but results are inconclusive due to the limitations of the methodology

4.7.1 The effects of *RAPSN* mutations on protein levels of rapsyn-EGFP

When overexpressed in heterologous cells, the V45M mutation significantly reduced the expression of rapsyn-EGFP compared to WT. N88K marginally reduced rapsyn-EGFP expression in HEK 293 but this difference was not detected in TE671 cells. This may be due to the limitations in detecting subtle differences by the semi-quantitative western blotting. Mutations in the E-box promoter region of *RAPSN* impair the transcriptional activities of *RAPSN* and reduce protein expression (Ohno et al., 2003). Therefore reduced expression in itself has been found to be a pathogenic property. It would be of interest to see whether patients who are homozygous or compound heterozygous with V45M or other null mutations show phenotypic similarities to patients with E-box promoter mutations. However, only very few of these patients have been identified to date, thus this analysis is not yet feasible.

V45M, N88K, R91L and A153T reduced the protein stability of rapsyn-EGFP, with V45M having the most dramatic effect. Increased turnover of rapsyn by proteasome-dependent degradation *in vivo* was demonstrated to impact on the formation and maintenance of AChR clusters (Luo et al., 2008). Hence the severe impairment in the expression level of rapsyn caused by V45M is likely to be a major cause for the reduced AChR clustering reported.

4.7.2 The *RAPSN* mutations affected the levels of AChR on myotube surfaces

The ^{125}I - α -BuTx binding assay was performed to provide ‘snap-shot’ information on the expression of AChR on myotubes expressing the different variants of rapsyn-EGFP. The *RAPSN* mutations investigated reside within the TPR domain of rapsyn, which was previously reported to be crucial for the metabolic stabilisation of AChR on membrane surfaces (Wang et al., 1999).

The expression of WT- or R91L-rapsyn-EGFP increased the level of AChR expression on myotubes surfaces compared to *Rapsn*-KO myotubes expressing EGFP, suggesting that these variants of rapsyn-EGFP may function in the insertion of AChR into the surface membrane, or may stabilise the receptors that are already inserted (Gervasio and Phillips, 2005; Losen et al., 2005). The results also indirectly suggest that the interaction between R91L-rapsyn-EGFP and AChR is not affected. This is consistent with the previous study that showed the mutation attenuates rapsyn self-association (Cossins et al., 2006). The mutant N88K-, V45M-, and A153T-rapsyn-EGFP led to the reduced expression of AChR on myotube surfaces, possibly due to their reduced expression and/or stability which attenuated their ability to stabilise the AChR in the surface membrane.

4.7.3 The *RAPSN* mutations reduce the stability of AChR clusters

Similar to ACh, carbachol induces AChR clusters disassembly by the calpain/Cdk5 signalling pathway (Chen et al., 2007; Lin et al., 2005). The rapsyn-calpain interaction has been found to suppress calpain activity, which in turn stabilises AChR clusters (Chen et al., 2007). The relative percentages of the number and total area of agrin-induced AChR clusters remaining on myotubes, after exposure to carbachol, expressing V45M-, N88K-, R91L- and A153T- rapsyn-EGFP at 1.5-6 hours are significantly less when compared to WT. The AChR clusters formed by mutant-rapsyn-EGFP were lost at a faster rate, which suggests they are less stable.

Differences in the carbachol-induced reductions in the average size of AChR clusters formed by WT- and mutant- rapsyn-EGFP were subtle. The histogram distribution revealed that for all variants, most AChR clusters formed were small ($2.5\text{-}7.5\mu\text{m}^2$), and the disassembly of AChR clusters likely reduced the size of small clusters to below the $2.5\mu\text{m}^2$ threshold that qualifies clusters for quantification. As such, the mean of AChR cluster size may not closely reflect the reduction in cluster size, and is less suited as a parameter to determine the stability of AChR clusters.

Analyses of the distribution of AChR clusters at 0 and 24 hours after carbachol treatment found that the proportion of AChR clusters present on myotubes expressing N88K-, R91L and A153T- rapsyn-EGFP were significantly reduced after exposure to carbachol. The findings suggest that larger AChR clusters formed by these mutant-rapsyn-EGFP were fragmented during disassembly, and to a lesser extent for AChR clusters formed by WT- or V45M- rapsyn-EGFP.

4.7.4 Do the *RAPSN* mutations influence the turnover of AChR after cluster disassembly?

The addition of carbachol to myotubes with/without excess FLaGrin caused significant reductions, of ~15% and 35% respectively, in the amount of ^{125}I - α -BuTx bound. Thus carbachol increased AChR loss, but the presence of excess FLaGrin stabilised AChR on myotubes surfaces. This is consistent with publications that have identified that agrin modulates AChR stability (Bezakova et al., 2001), and protects against the cholinergic dispersal of AChR clusters (Misgeld et al., 2005). Furthermore, the half-lives of clustered AChR were estimated to be ~4 times longer than non-synaptic AChR *in vivo* (Akaaboune et al., 2002), suggesting that clustered AChR are metabolically more stable. A mechanism of this effect of FLaGrin is through rapsyn expression levels (Brockhausen et al., 2008), where an increase in the expression of rapsyn increases AChR packing at clusters and reduces AChR turnover (Gervasio and Phillips, 2005; Misgeld et al., 2005).

There was no significant difference identified when the effects of *RAPSN* mutations on AChR loss were compared, possibly due to the limitations of the ^{125}I - α -BuTx binding to specifically detect clustered AChR. Although not statistically significant, after 6 hours of carbachol, myotubes expressing A153T-rapsyn-EGFP showed a bigger reduction in the levels of ^{125}I - α -BuTx bound compared to WT. It may be that A153T attenuates interactions between rapsyn-EGFP and AChR, and affects AChR stability. A more detailed analysis of the effect of carbachol on the turnover of clustered AChR could be achieved by live imaging AChR clusters labeled with α -BuTx-594.

4.7.5 Establishing parameters for the analysis of fluorescence recovery after photobleaching (FRAP) for AChR clusters

Investigations into the mobility of AChR on myotube surfaces started in the 1970s, and it was identified that diffusely distributed AChR are mobile, but clustered AChR are less so (Axelrod et al., 1976b). Later it was shown that AChR are continuously exchanged between the clustered and perisynaptic regions in adult NMJ, and individual AChR may reside at any site within clusters for approximately 8 hours (Akaaboune et al., 1999; Akaaboune et al., 2002). Single-molecule tracking experiments using muscle and heterologous cells have demonstrated that rapsyn restricts the mobility of AChR independently from other postsynaptic components (Piguet et al., 2011). FRAP allows the analysis of two-dimensional lateral mobility of fluorescently labelled proteins on live cells (Axelrod et al., 1976a). Rapsyn-EGFP has been confirmed to function like endogenous rapsyn. The presence of the EGFP moiety at the C-terminus of rapsyn does not affect AChR clustering, the function or removal rate of AChR either in cell culture or *in vitro* (Bruneau and Akaaboune, 2007; Bruneau and Akaaboune, 2010).

Spontaneous contraction of some myotubes was found to shift the localisation of the cluster under investigation, which can cause misleading results in FRAP. To overcome this problem, the observation period was kept short (~1 minute) and a measurement box that is double the size of the bleached region was used to obtain the mean fluorescence intensity. This effectively reduced the non-specific ‘noise’ in the data obtained, but also compromised the precision of the FRAP data, as the mean fluorescence intensities measured were not specific to the clustered regions. In future, it would be desirable to inhibit the contraction of myotubes. Increased

potassium ion concentration in the culture medium or the application of tetrodotoxin, a paralytic agent that antagonises voltage-gated sodium channel, to myotubes was reported to cause the immediate cessation of spontaneous contraction (Bandman and Strohman, 1982; Walker and Strohman, 1978). The suitability and efficacy of these options in reducing myotube contractions of the cells could be explored.

The lateral migration of rapsyn-EGFP and the insertion of newly synthesised rapsyn-EGFP from intracellular locations can both contribute towards FRAP. As the current protocol utilises a very short observation period after photobleaching and newly synthesised AChR are not labeled with α -BuTx-594, the recovery of α -BuTx labelled AChR is more likely to have originated from the lateral migration of the fluorescently tagged/labeled proteins.

FRAP of rapsyn-EGFP within clusters was previously found to reach ~80% of its original intensity 6 hours after photobleaching (Bruneau and Akaaboune, 2007). A goal would be to achieve live imaging and FRAP analysis of AChR clusters for a similar duration to confirm experiments that suggested the *RAPSN* mutations increase the mobility of rapsyn and/or AChR within clusters.

4.7.6 The *RAPSN* mutations alter the mobility of rapsyn-EGFP and AChR within clusters on myotubes

The fluorescence recovery of rapsyn-EGFP (~10% for WT) or α -BuTx-594 labelled AChR (~4% for WT) after photobleaching, within ~1 minute, was small. This is consistent with a previous study that reported little recovery of rapsyn-EGFP and AChR within an AChR cluster 5 minutes after photobleaching (Gervasio and Phillips, 2005). The faster recovery of rapsyn-EGFP is in agreement with the halftime of the recovery of rapsyn-EGFP at clusters on C2C12 myotubes (~1.5hours), which was found to be 3 times faster than AChR (Bruneau and Akaaboune, 2007). The concordance of the presented FRAP data and published results suggests that the results reliably reflect the effects of *RAPSN* mutations on the dynamics of rapsyn-EGFP and AChR within clusters on myotubes.

The mutation A153T increased the mobility of rapsyn-EGFP within clusters. Impairment in rapsyn self-association and/or the interaction between rapsyn-EGFP and the cytoskeleton may account for the increased movement of rapsyn-EGFP observed. Since rapsyn is directly involved in restricting AChR mobility within clusters (Piguet et al., 2011), the recovery of AChR was also increased from clusters formed by A153T-rapsyn-EGFP, suggesting the AChR-rapsyn-EGFP complex could be free from cytoskeletal constraints. The increased mobility of AChR was also observed on clusters formed by V45M- and N88K- rapsyn-EGFP, suggesting in these cases that the mutations impaired rapsyn-AChR interactions. An alternative explanation could be for the reduced expression and stability of these mutant-rapsyn-EGFP variants to reduce AChR clustering, therefore increasing AChR mobility.

However, the results at this stage should be treated with caution as intense laser illumination can dissociate some α -BuTx-594 from labelled AChR, a phenomenon known as photo-unbinding (Akaaboune et al., 2002). It has been reported that displaced fluorescent ligands can rebind to AChR and cause ~11% false recovery in FRAP experiments (Akaaboune et al., 2002). There is evidence that suggests photo-unbinding did not contribute significantly to the presented results. Firstly, the FRAP for AChR was much lower than the suggested false recovery caused by photo-unbinding. Secondly, the preliminary results (not shown) and a previous report (Gervasio and Phillips, 2005) found no recovery when clusters of rapsyn-EGFP and AChR on paraformaldehyde fixed cells were photobleached. Thirdly, rapsyn-EGFP is a recombinant fusion protein that fluoresces, and the high intensity light is unlikely to split the rapsyn-EGFP protein. Regardless of this, the influences of photo-unbinding in the current setup for FRAP analysis could be assessed in the future.

Studies on the AChR-rapsyn complexes suggested that AChR are connected by up to 3 rapsyn bridges, and approximately 75% of the AChR presented in clusters were found in groups of 2-14 connecting AChR (Zuber and Unwin, 2013). It was proposed that AChR and rapsyn undergo cycles of transient interactions, and unconnected AChR are trapped between other immobilised AChR within clusters (Zuber and Unwin, 2013). Supporting the hypothesis, more mobile AChR, with less restricted mobility refinement domains (~120nm), was found on *Rapsn*-KO myoblasts in comparison to C2C12 myoblasts (~50nm) (Piguet et al., 2011). If the hypothesis is valid, increased mobility of AChR may increase the chances for AChR to escape into perisynaptic regions and become internalised (Akaaboune et al., 1999; Akaaboune et al., 2002).

Chapter 5

The pathogenic mechanisms of a mutation in the AChR delta subunit gene that was found to mimic *RAPSN*-CMS

5.1 Introduction

The analysis of the effects of *RAPSN* mutations on AChR clustering focused on the functional importance of rapsyn, but AChR plays more than a passive role in its co-clustering with rapsyn on the postsynaptic membrane. It has been shown that the trafficking of rapsyn from the Golgi complex to the plasma membrane requires the expression of functional AChR (Park et al., 2012). AChR-null variants of C2 myotubes do not form clusters of rapsyn or other postsynaptic scaffolding proteins after stimulation with agrin (Marangi et al., 2001). It is notable that overexpression of rapsyn bypasses this mechanism and allows rapsyn clustering on the plasma membrane (Cossins et al., 2006; Ohno et al., 2002; Park et al., 2012). The dissipation of AChR, caused by exposure of the receptors to a low-powered laser light, from AChR clusters on C2C12 myotubes resulted in the loss of rapsyn and other scaffold proteins at the site, suggesting that AChR modulates the maintenance of the clustering of intracellular scaffold (Bruneau et al., 2008). In *Caenorhabditis elegans*, the stability of RPY-1, a putative homologue of rapsyn, is regulated by the cytoplasmic loop of a non- α -nAChR subunit, UNC-29 (Nam et al., 2009). Mutations in the AChR subunit genes may also result in AChR deficiency due to attenuated AChR clustering at the NMJ.

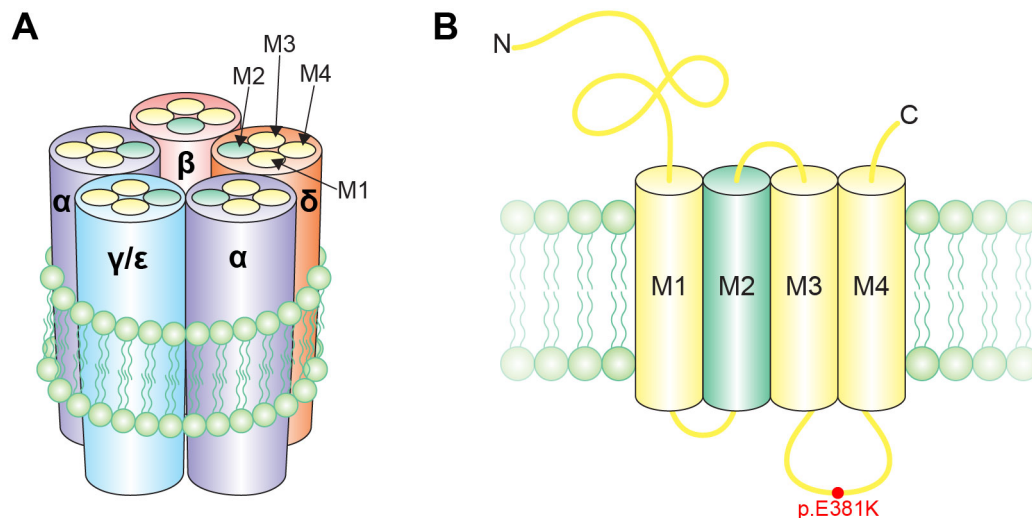


Figure 5-1 Schematic representation of the AChR and the location of the *CHRND* mutation E381K

(A) The muscle AChR consists of 4 different subunits: $\alpha_2\beta\delta\gamma$ or $\alpha_2\beta\delta\epsilon$ for the foetal and adult pentamers respectively. **(B)** Each of the subunits contains an N-terminal extracellular domain, 4 transmembrane domains (M1-M4), and a large cytoplasmic loop between M3 and M4, in which the *CHRND* E381K mutation resides (red)

The muscle AChR consists of 4 distinct yet homologous subunits: $\alpha_2\beta\delta\gamma$ and $\alpha_2\beta\delta\epsilon$ for foetal and adult forms respectively. Each subunit consists of an N-terminal extracellular domain, followed by 4 transmembrane domains (M1-4), and a long cytoplasmic loop between M3-M4 (Figure 5-1), which is required for the interaction between rapsyn and AChR (Huebsch and Maimone, 2003). Studies that specifically investigated this region using chimeric constructs showed that the cytoplasmic loops of the AChR subunits interacts with rapsyn with different affinities, and the δ -subunit was suggested to be not involved in the process (Lee et al., 2009).

A common pathogenic mechanism of mutations in the AChR subunit genes is through endplate AChR deficiency, which usually results from impaired expression, assembly or trafficking of AChR to the membrane surface (Finlayson et al., 2013; Ohno and Engel, 2002). Although *RAPSN*-CMS also causes AChR deficiency,

clinical presentations of patients with *RAPSN* mutations are distinctly different from patients that harbour *CHRNE* mutations (Burke et al., 2004).

A patient with clinical features that are suggestive for *RAPSN*-CMS did not carry any *RAPSN* mutations, but was found to harbour 2 mutations in the AChR δ -subunit gene (*CHRND*): a chromosomal microdeletion Δ 2.2kb that results in a non-functional allele where half of exon 8 and the entire exon 9 of the gene are lost, and a missense mutation p.Glu381Lys (E381K) (Muller et al., 2006b). The phenotype of the patient is therefore determined by E381K, which resides within the cytoplasmic loop of the δ -subunit (Figure 5-1). Functional analysis, by overexpression of AChR with or without rapsyn in HEK 293 cells suggested that E381K impairs rapsyn-AChR co-clustering and mildly reduces the cell surface expression of AChR (Muller et al., 2006b). The defective rapsyn-AChR interaction was found to be specific to the δ -subunit as the same mutation introduced to the ϵ -subunit did not affect rapsyn-AChR co-clustering (Muller et al., 2006b). Therefore the data on E381K suggests a unique, but yet-to-be defined role for the δ -subunit that is associated with rapsyn functions.

5.1.1 AChR clustering on WT-C2C12 overexpressing δ E381K

To confirm that the E381K mutation impairs agrin-induced AChR clustering by rapsyn, the mutant δ -subunit was overexpressed in C2C12 cells. However, the assembly of AChR incorporating the human δ -subunit could be outcompeted by the endogenous mouse AChR subunits expressed in C2C12 cells. Since the p.E381 residue and its proximal region are conserved between human and mouse δ -subunits, and *Chrnd* shares 88% homology to the *CHRND* overall, the experiment was performed by the overexpression of mouse δ -subunits in C2C12 cells. (Figure 5-2)

Range 1: 1 to 496 [Graphics](#) ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
889 bits(2296)	0.0	Compositional matrix adjust.	435/496(88%)	469/496(94%)	0/496(0%)
mouse 1		LNEEQRLIQHLFNEKGYDKDLRPVARKEDKVDVALSLTSLNLISLKEVEETLTITNVWIDH			60
human 1		 E R Q N .. E H .. ES A E ..			60
mouse 61		AWVDSRLQWDANDFGNITVLRLPDMVWLPEIVLENNNDGSFQISYACNVLVYDSGYVTW			120
human 61		G .. T .. N .. K .. N .. EE S S HY .. F .. Y ..			120
mouse 121		LPPAIFRSSCPISVTYFPFDWQNC SLKFSSLYTAKEITLSLKQEEENRSYPIEWIID			180
human 121		 DAKE .. T .. V			180
mouse 181		PEGFTENGWEIVHRAAKLNVDPSVPMDSTNHQDVTFYLIIRRKPLFYIINILVPCVLIS			240
human 181		 P .. RV RA .. L .. PSR .. I			240
mouse 241		FMINLVFYLPGDCGEKTSVAISVLLAQSVFLLLSKRLPATSMALPLVGKFLFLFGMVLVT			300
human 241		.. V A .. S I			300
mouse 301		MVVVICVIVLNIHFRTPSTHVLSEGVKKFLETLPKLLHMSRPAEEDPGPRALIRRSSSL			360
human 301		 L E DG .. S .. G .. V			360
mouse 361		GYICKAEYFSLKSRSDLMFEKQSERHGLARRLTTARRPPASSEQVQQLFNEMKPAVDG			420
human 361		.. S L A L			420
mouse 421		ANFIVNHMRDQNSYNEEKDNWNQVARTVDRLCLFVVTPVMVGTAWIFLQGVYNQPPLOP			480
human 421		 N S .. R P ..			480
mouse 481		FPGDPFSYSEQDKRFI			496
human 481		 Y .. NV			496

Figure 5-2 Alignment of amino acid sequences for the AChR δ -subunit in humans and mice

The amino acid sequences of human (*RefSeq: NM_000751/NP000742*) and mouse (*RefSeq: NM_021600/NP_067611*) AChR δ -subunit were analysed by Protein BLAST. The alignment of the amino acid sequences minus the signal peptide for the protein, with mismatches shown in red, shows the sequences share 88% identities. The p.E381 (blue) and neighbouring residues are conserved between human and mouse.

δ WT or δ E381K, together with the EGFP expression marker that is not tagged to the protein, were overexpressed in C2C12 myotubes by retroviral infection to investigate the pathogenic effects of E381K on agrin-induced AChR clustering. Uninfected C2C12 cells and cells that only overexpress EGFP were also analysed as additional controls to evaluate the reliability of this system.

Western blot analysis of the whole cell lysate of myotubes confirmed the overexpression of δ WT or δ E381K in C2C12, and the representative fields of agrin-induced AChR clusters formed on the myotubes are shown in Figure 5-3. The quantified results are shown in Figure 5-4.

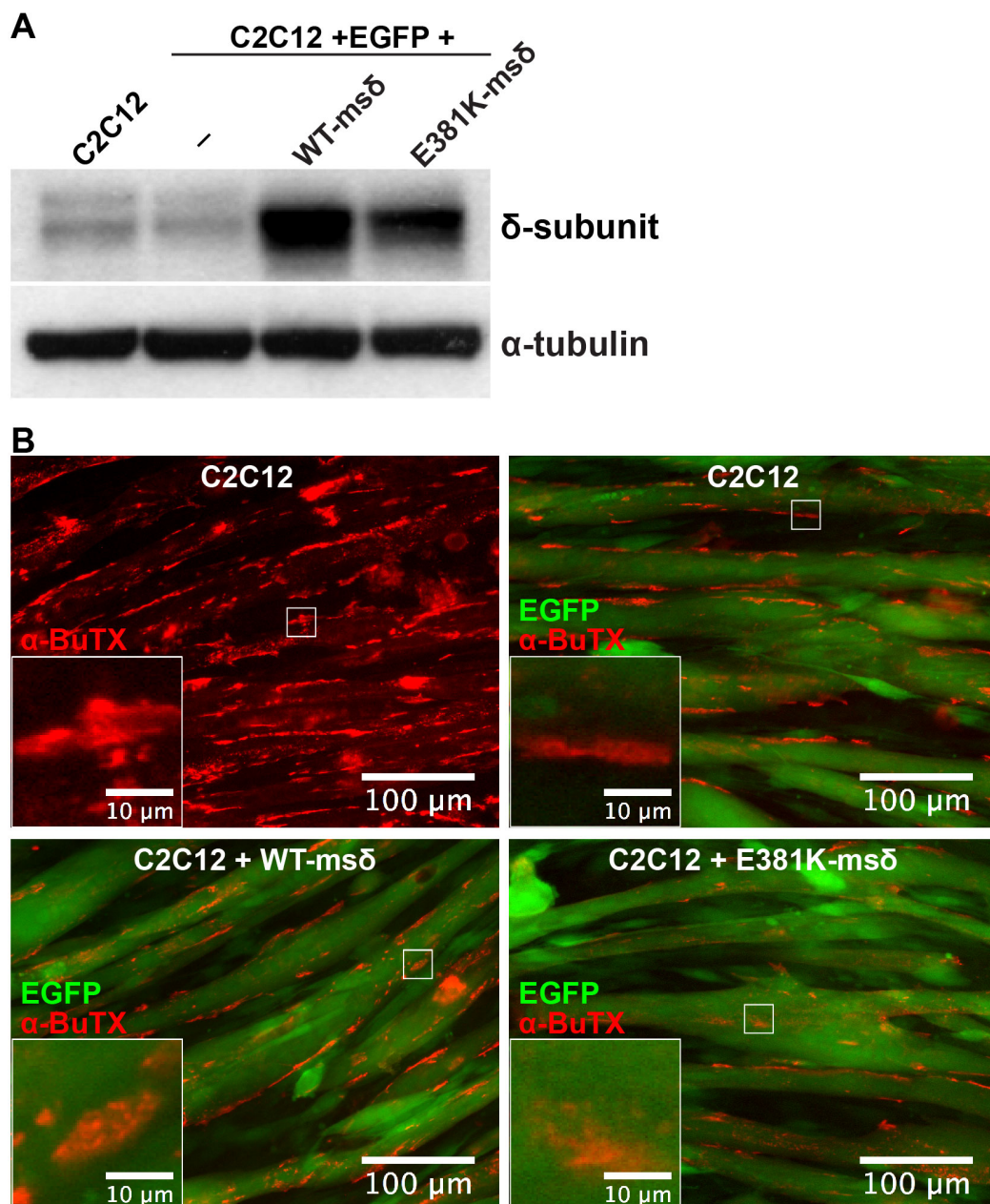


Figure 5-3 Representative microscopic fields from AChR clustering assay on C2C12 myotubes overexpressing WT- or E381K- ms delta subunit of AChR

WT- or mutant E381K- ms- δ subunit of AChR was overexpressed in C2C12 cells by retroviral infection. An expression marker EGFP that is not tagged to the δ subunit confirmed the overexpression of the proteins. The C2C12 myoblasts were differentiated into myotubes and incubated with 1 in 100 FLagrin to induce AChR clustering. Untreated C2C12 and C2C12 that overexpressed EGFP were included as controls.

(A) Western blot analyses with antibodies against δ -subunit of AChR and α -tubulin confirmed the overexpression of the δ -subunit

(B) Representative images of AChR clusters labelled with α -BuTx-594

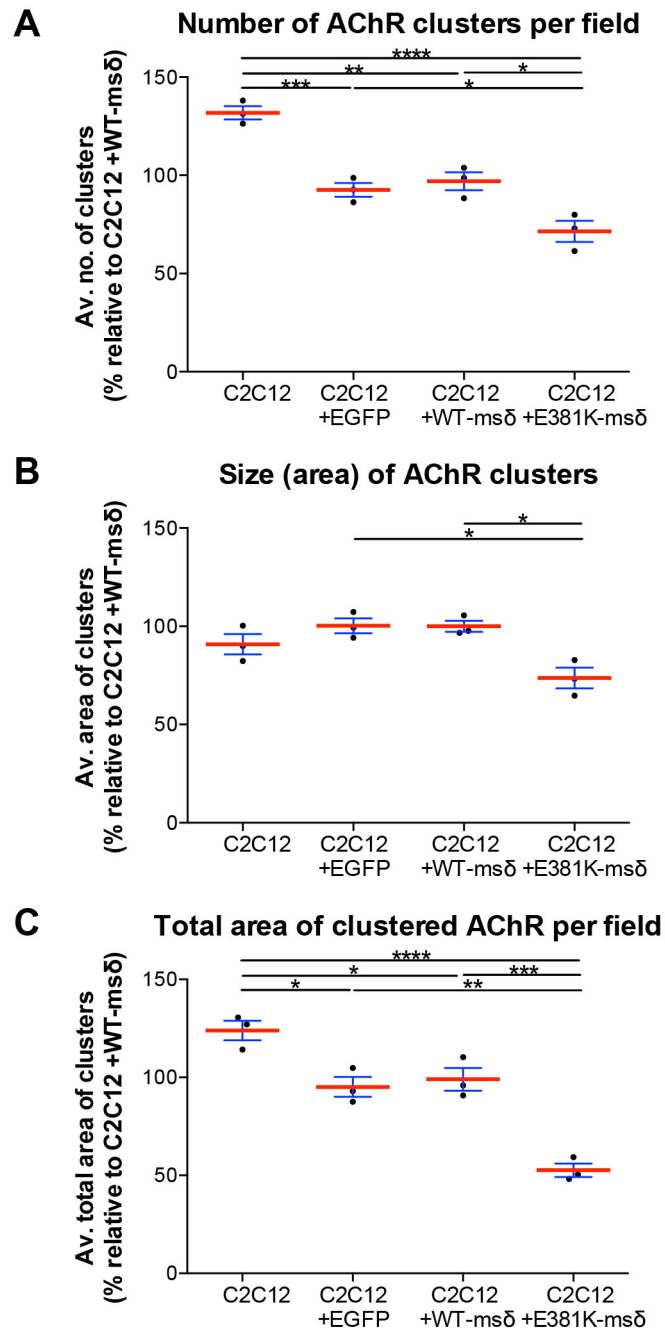


Figure 5-4 Quantified data of AChR clustering on C2C12 myotubes overexpressing WT- or E381K- msδ subunit of AChR

Myotubes overexpressing WT- or mutant-E381K- δ -subunit of AChR were stimulated with FLagrin for AChR clustering, and AChR clusters were labelled with α -BuTx-594. For each variant, 60 images from 3 experiments were analysed.

Data points represent the mean of data from 20 images for each condition, and the results are normalised to the clustering properties of myotubes overexpressing WT-msδ (mean percentage $\pm$ SEM). Asterisks ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05. One-way ANOVA and Tukey's multiple comparisons test.

The number and total area of AChR clusters formed on myotubes overexpressing EGFP were significantly less than untreated C2C12 myotubes, suggesting a possible detrimental effect of the retroviral infection to cell behaviour. Regardless, myotubes overexpressing δ WT+EGFP formed a similar number of AChR clusters to myotubes expressing EGFP only, and the clusters formed were similar in average size and total area per microscopic field. Hence AChR clusters formed in the presence of δ E381K can be compared to myotubes overexpressing δ WT to evaluate the pathogenic effects.

In comparison to myotubes overexpressing δ WT, the number, size and total area of AChR clusters per field were significantly reduced on myotubes overexpressing δ E381K. Therefore E381K impair AChR clustering, and its underlying mechanisms warrant further investigation. However, the presence of endogenous δ WT in C2C12 may hinder data interpretation for the functional effects of *Chrnd* E381K. A better model system is a cell line that only expresses the δ E381K subunit.

The phosphorylation of Ser³⁷⁸, Ser³⁹³ and Ser⁴⁵⁰ in the δ -subunit by protein kinase C (PKC) was found to be accompanied by the dispersal of AChR clusters formed on myotubes (Nimmual et al., 1998). It is possible for the δ -subunit to have important roles in regulating the stability of AChR clusters at the NMJ. The mutation E381K is only 3 amino acids away from the Ser³⁷⁸ phosphorylation site, and therefore could have related pathogenic effects on agrin-induced AChR clustering analysis. Thus investigation into the clustering of AChR assembled with δ E381K is an opportunity to study the effects of defective AChR functioning on AChR clustering.

5.1.2 Aims

Although the functions of rapsyn and AChR are known to be interdependent, the precise sites of interaction between rapsyn and AChR at the NMJ have yet to be identified. The cytoplasmic loop of the δ -subunit did not interact with rapsyn in chimeric protein experiments (Lee et al., 2009), but the *CHRND* mutation E381K phenotypically mimics *RAPSN*-CMS, and is found to cause AChR deficiency due to reduced AChR clustering by rapsyn (Muller et al., 2006b). It is possible for the δ -subunit to have a role in the interaction process with rapsyn that can only be identified when the native subunit is assembled in AChR. Conversely, despite the results from artificial *in vitro* systems, it may be that the δ -subunit is crucial for the AChR interaction with rapsyn. The proximity of E381K to the Ser³⁷⁸ PKC phosphorylation site suggests a possible pathogenic effect on the stability of AChR clusters. To date, E381K is the only *CHRND* mutation found to have pathogenic effects associated with defective AChR clustering. This chapter aims to further our understanding of the underlying mechanisms of the E381K mutation.

5.2 Introduction to the CRISPR-Cas9 system for genome engineering

The Type II clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 system, derived from *Streptococcus pyogenes*, provides a platform for targeted genome engineering (Cho et al., 2013; Jinek et al., 2012; Ran et al., 2013b). The system consists of the nuclease Cas9 and a chimeric single-guide RNA (sgRNA) that is generated by the fusion of a target-specific CRISPR RNA (crRNA) and a target-independent transactivating CRISPR RNA (tracrRNA) (Jinek et al., 2012). Cas9 can be directed to target DNA by a complementary 20-nucleotide sequence that is hybridised to the 5' of sgRNA (crRNA), and induce a double-stranded DNA break (DSB). One requirement for Cas9 cleavage is a 5'-NGG protospacer adjacent motif (PAM) downstream to the genomic target sequence (Jinek et al., 2012). At the site of DSB, the DNA undergoes damage repair by either non-homologous end joining (NHEJ) or homology-directed repair (HDR). The NHEJ mechanism is error-prone and can be harnessed to mediate gene knockouts, as indels in the coding exon can result in frameshift mutations and premature stop codons (Mehrabian et al., 2014; Zheng et al., 2014). On the other hand, the presence of a repair template can promote HDR to introduce specific modifications at the target locus (Chen et al., 2011).

A mutant (D10A) nickase version of Cas9, Cas9n, induces single-stranded DNA breaks, and can be directed by a pair of sgRNA to opposite strands of the target DNA to mediate a DSB (Jinek et al., 2012; Ran et al., 2013a; Ran et al., 2013b). The double nicking approach offers higher target specificity (Ran et al., 2013a), and DNA repair preferentially occurs via the HDR pathway (Cong et al., 2013) (Figure 5-5).

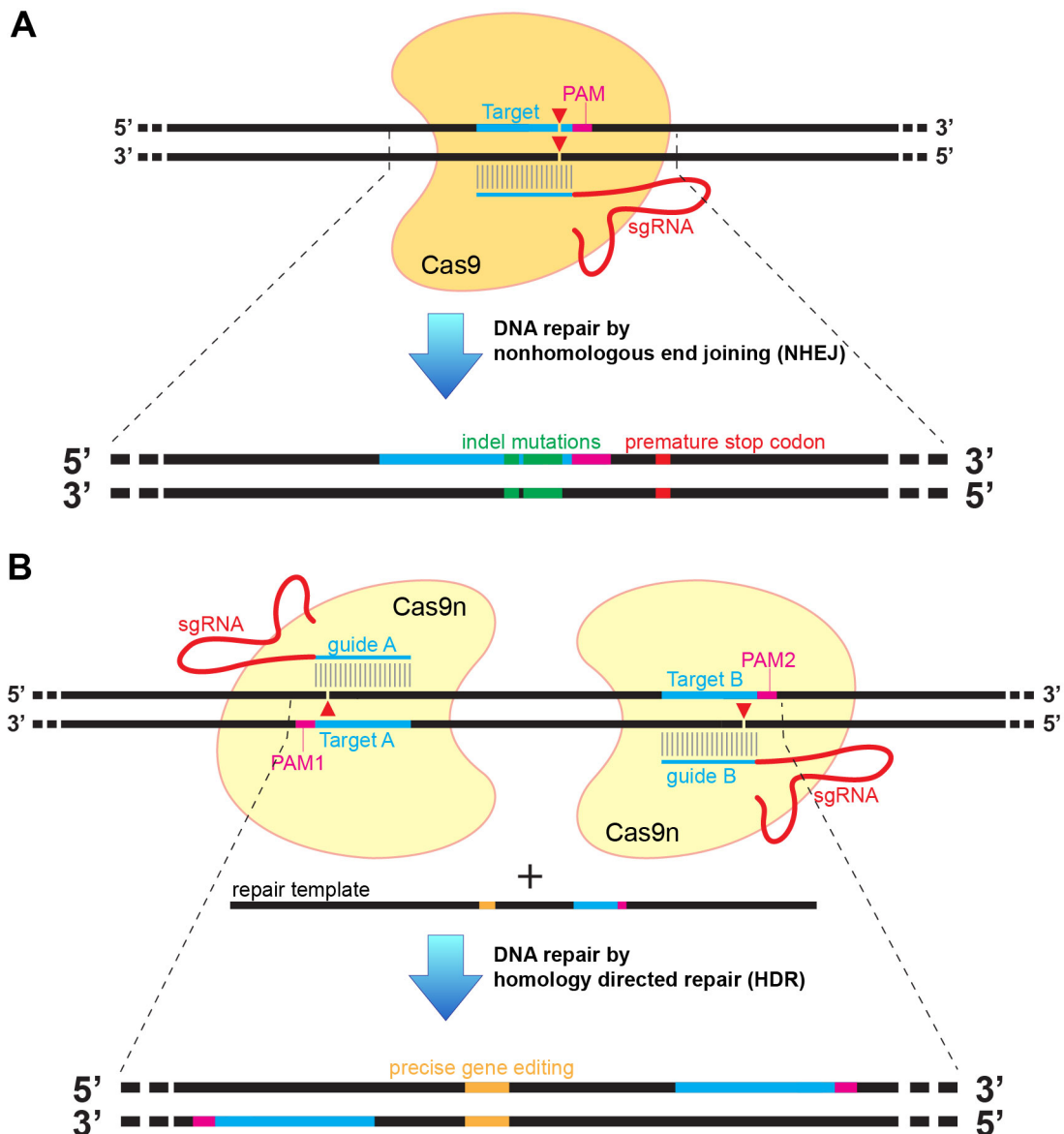


Figure 5-5 Schematic of the RNA-guided Cas9 and Cas9n nuclease

(A) The Cas9 nuclease is directed to the target site by the sgRNA scaffold connected to a 20-nucleotide guide sequence that is complementary to the target sequence. Cas9 mediates a double stranded break (DSB) at 3 nucleotides upstream to the required protospacer adjacent motif (PAM). The DSB can be repaired by nonhomologous end joining (NHEJ), which is error prone and can give rise to indels.

(B) The D10A mutant nickase version of Cas9 (Cas9n) nicks one strand of the target DNA. A selected pair of guide sequences on opposite strands would lead to double nicking and generates a DSB. Homology directed repair (HDR)-mediated gene editing with a single-stranded oligonucleotide repair template that encodes the desired changes flanked by complementary genomic sequences allows specific gene modifications. Of note, NHEJ and HDR can both occur at DSB mediated by the WT-Cas9 or Cas9n double nicking system

5.3 Establishing the protocol for CRISPR-Cas9n mutagenesis of C2C12 cells

To generate C2C12 clones that harbour the mutation E381K in *Chrnd*, the double nicking strategy by Cas9n, together with a single-stranded oligonucleotide repair template (GAG>AAG substitution for the codon E381K in the centre flanked by 60 nucleotides of the genomic sequence on either side) were used to promote the HDR-mediated gene editing. The CRISPR Design Tool (<http://crispr.mit.edu>) was used to identify a suitable pair of guide oligonucleotide sequences, guides A and B, that target Cas9n to the proximal loci of E381 in *Chrnd* at opposite strands of the genomic DNA (Figure 5-6 A). The chosen combination of guides A and B was predicted to have no off-target effects in the mouse genome. In addition, the 2 PAM sites were silently mutated on the repair template to avoid repeat nicking of homologous recombined genomic DNA (PAM1: CCC>GAG, PAM2: TGG>TAG).

The CRISPR plasmid: pX335-U6-Chimeric_BB-Cbh-hSPCas9n(D10A) was provided by Dr. Joey Riepsaame at the Weatherall Institute of Molecular Medicine, and it contains an EGFP and a neomycin resistance gene to allow for the selection of transfected cells. Guides A and B were cloned into pX335-U6-Chimeric_BB-Cbh-hSPCas9n(D10A) so that the guide sequences are linked to the sgRNA scaffold for Cas9n. Subsequently, the prepared CRISPR plasmid was co-transfected with the repair template into C2C12 myoblasts by electroporation. Twenty-four hours after transfection, the transfected C2C12 myoblasts were selected by treatment with Geneticin G418 (1mg/ml) for 48 hours and then limiting dilution was performed to isolate individual clonal cell lines (Figure 5-6 B).

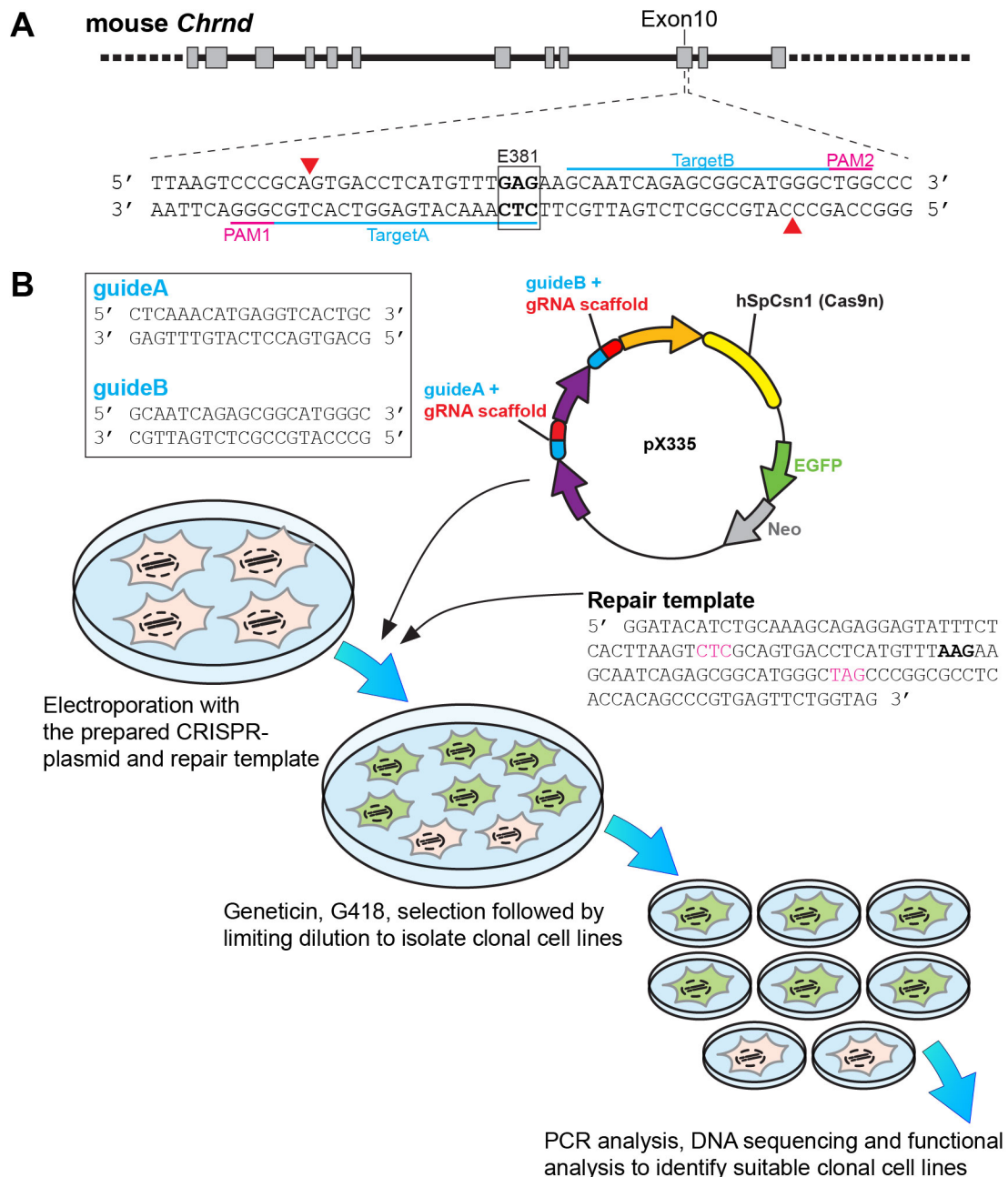


Figure 5-6 Overview of CRISPR-Cas9n mutagenesis for *Chrnd* E381K in C2C12

(A) The 3' ends of the 20bp target sequences (targets A and B, blue) are immediately followed by the 5'-NGG PAM (pink) which directs Cas9n to the target sites to double nick (red triangles) genomic DNA and to generate a DSB in proximity to the E381 loci.

(B) Sequences of guides A and B were cloned into pX335 so that they each precede a sgRNA scaffold. The prepared plasmid and single-stranded repair template were co-transfected into C2C12 myoblasts by electroporation. The repair template contains the mutation GAG>AAG for E381K (bold), and silently mutated PAM sites (pink) to prevent repetitive nicking of DNA. The transfected C2C12 myoblasts were incubated with Geneticin for 48 hours to select for successfully transfected cells. Subsequently clonal cell lines were isolated by limiting dilution and were expanded for analysis.

To screen for C2C12 clones that harbour the *Chrnd* mutation, E381K, DNA was extracted from the cells and subjected to PCR analyses with a pair of primers that was designed to specifically amplify the region of genomic DNA that includes the CRISPR target sites. The PCR products were then resolved by electrophoresis on agarose gels and a single band that is 745 base pairs (bp) in size is expected for DNA that is WT or that harbours the mutation E381K. Hence DNA from C2C12 clones that yielded multiple PCR products or products that are bigger/smaller than ~750bp are likely to have been repaired by non-homologous end joining and to have generated unwanted indels, e.g. insertion/deletions. Since the primary aim for the CRISPR/Cas-9 mutagenesis is to generate C2C12 clonal cell lines that harbor the *Chrnd* mutation E381K, only PCR bands that were sized ~750bp were gel purified and analysed further to confirm the presence of the mutation (Figure 5-7 A).

The silent mutation TGG>TAG of PAM2 generates a restriction digest site, NheI, that is not present on WT PAM2 (Figure 5-7 B). The NheI restriction digest on the 745bp PCR product encompassing the CRISPR target sites enables the identification of clones that had been repaired by homology-directed repair with the repair template and harbor the mutated PAM2. The expected products from the NheI restriction digestion are 433 and 312bp in size and a successful restriction digest suggests the likelihood for C2C12 clones to harbour the mutation E381K (Figure 5-7 C). As the final step, the PCR products from clones that harbor the mutated PAM2 were analysed by DNA sequencing to verify the genotype of their originating clonal cells.

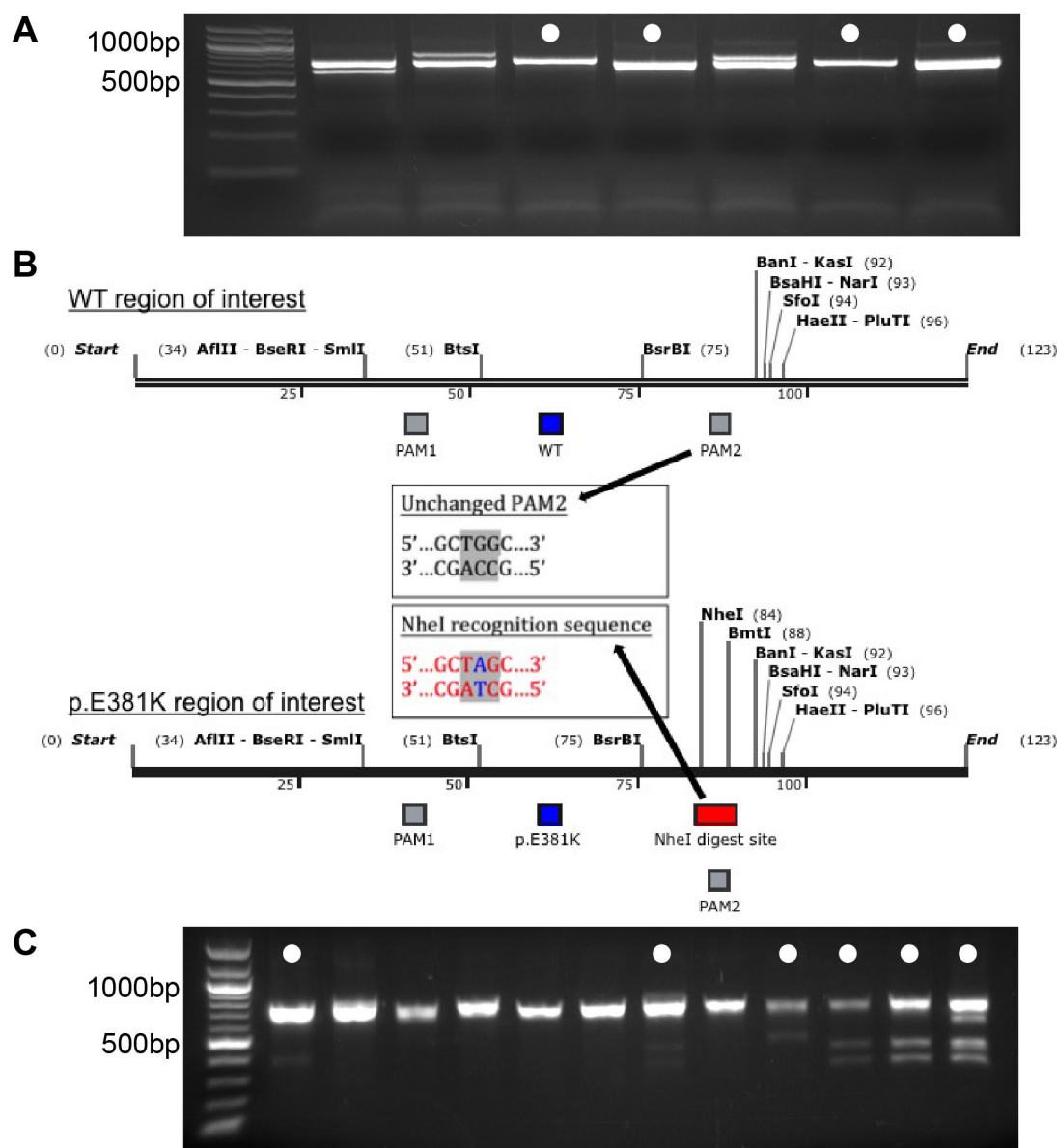


Figure 5-7 Screening for Chrnd E381K mutant C2C12 clones

(A) PCR products encompassing the CRISPR target sequences were resolved on 1% agarose gel to check for the expected single band product that is 750bp in size (white dots). The DNA of cells that yielded multiple PCR bands are likely to harbour unwanted indel and were not analysed further.

(B) The silent mutation at PAM2 yields the NheI recognition sequence that is not present on WT PAM2. (The region of interest represents the region of genomic DNA that is complementary to the repair template, i.e. 123 nucleotides)

(C) The PCR products (single band at 750bp) were gel purified and digested with NheI. The presence of NheI digested products (433bp and 312bp) suggests the DNA to have repaired via HDR with the repair template and harbours the mutated PAM2 (white dots). Therefore PCR products from these clones were subjected to DNA sequencing to confirm the genotype.

5.4 Controls for the process of CRISPR mutagenesis of C2C12 cell line

The CRISPR plasmid lacking guide oligonucleotide sequences was co-transfected with the repair template into C2C12 myoblasts. In this case, the genomic DNA of transfected cells should not be nicked by Cas9n and therefore should retain their WT-*Chrnd* genotype. DNA from 12 clonal cell lines (control 1-12) was extracted and analysed by PCR. A single band of PCR product was detected for all of the clones, and subsequently they were analysed by DNA sequencing, which confirmed their WT-*Chrnd* genotype (Figure 5-8 A).

The 12 control clonal cell lines were differentiated into myotubes and agrin-induced AChR clustering was assessed to confirm their suitability for experimental comparisons. However, only 8 control cell lines differentiated fully into myotubes. Two of the control cell lines did not differentiate and 2 differentiated partially and formed AChR clusters upon agrin stimulation. Moreover, the morphology of AChR clusters formed on myotubes varied (Figure 5-8 B).

To identify appropriate control cells lines, 5 control clones (controls 1, 2, 3, 5 and 11) were analysed further (Figure 5-9). Controls 1, 2, 5 and 11 were able to differentiate fully into myotubes, but displayed variable AChR clustering. Control 3 differentiated partially, and it was included to see if its ability to differentiate would recover with time. The protein expression of δ -subunit, cell surface AChR expression and AChR clustering were compared to age-matched C2C12 cells that were not treated with the CRISPR-Cas9n protocol.

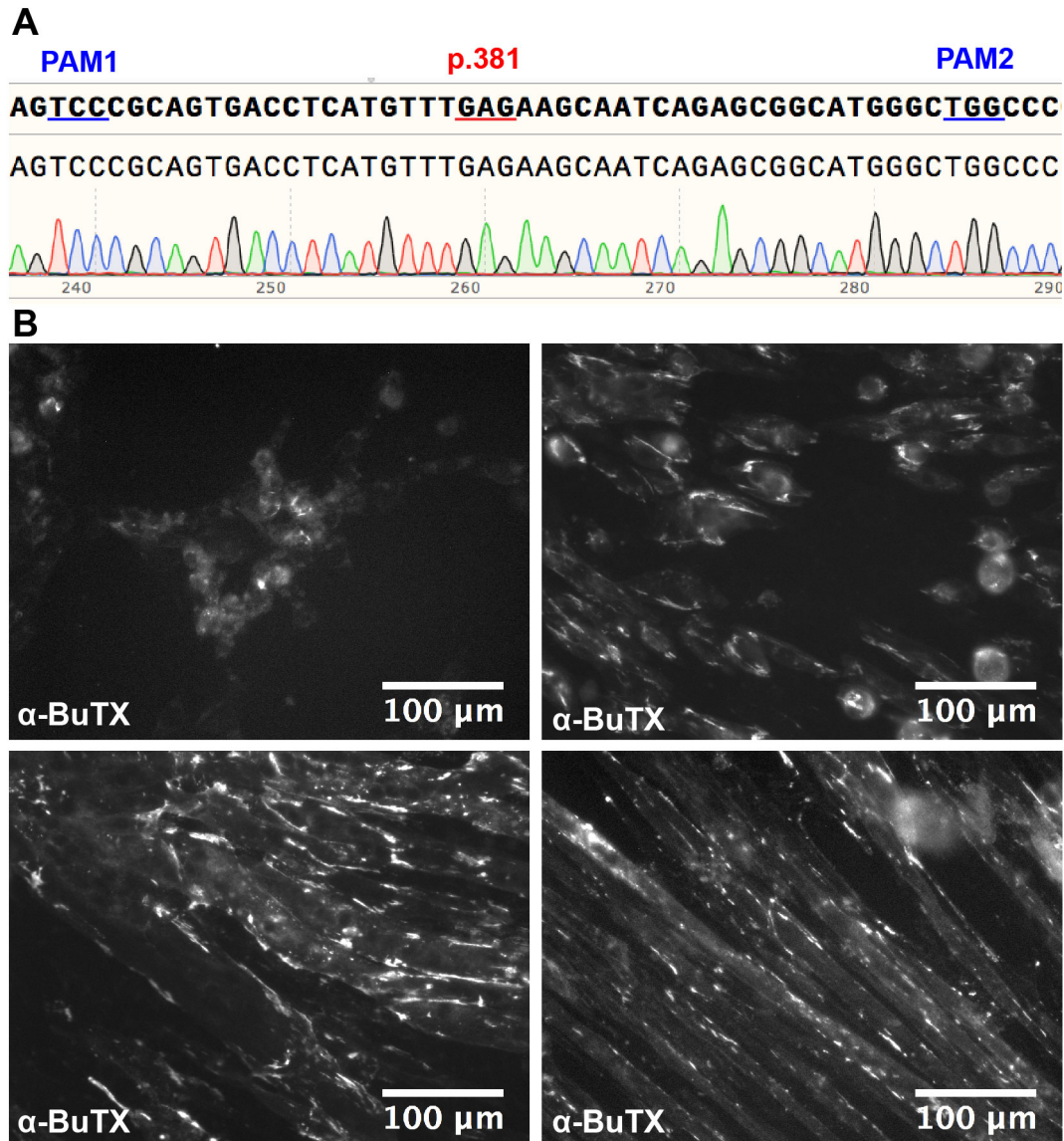


Figure 5-8 Generation of control C2C12 clonal cell lines

CRISPR plasmid lacking guide oligonucleotides was co-transfected into C2C12 myoblasts with the repair template. Subsequently the clonal cell lines were isolated by limiting dilution and were expanded. **(A)** DNA from 12 clones was analysed by sequencing and confirmed their WT δ -genotype.

The control cell lines were differentiated into myotubes and incubated with FLaGrin for 16 hours and AChR clusters formed were labeled with α -BuTx-594. **(B)** Example microscopic fields of clones that did not differentiate, partially differentiated or fully differentiated with AChR clusters formed.

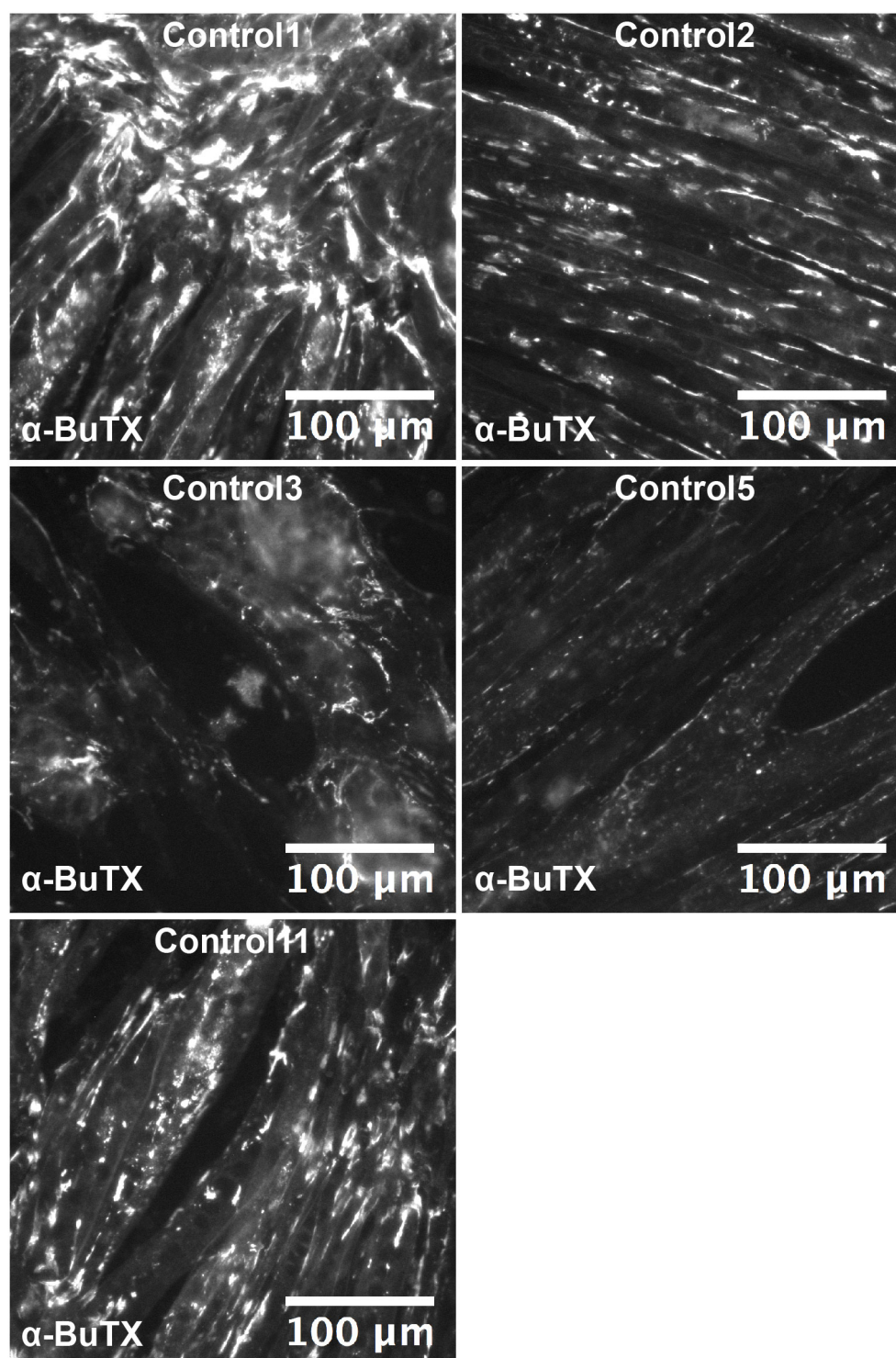


Figure 5-9 AChR clustering on myotubes of control clonal cell lines that are potentially useful and subjected to further analyses

The control cell lines were differentiated into myotubes and incubated with FLaGrin for 16 hours and the AChR clusters formed were labeled with α-BuTx-594. Controls 1, 2, 3, 5, and 11 were selected to evaluate their suitability for experimental comparisons.

Example images represent part of a microscopic field.

5.4.1 Protein expression of AChR δ -subunit in the myotubes of control C2C12 clonal cell lines

Western blotting analysis with antibodies against the AChR δ -subunit and α -tubulin was performed on whole cell lysates prepared from differentiated myotubes of untreated C2C12 and CRISPR controls. The ratios of δ -subunit: α -tubulin were normalised to expression levels of untreated C2C12 myotubes (Figure 5-10).

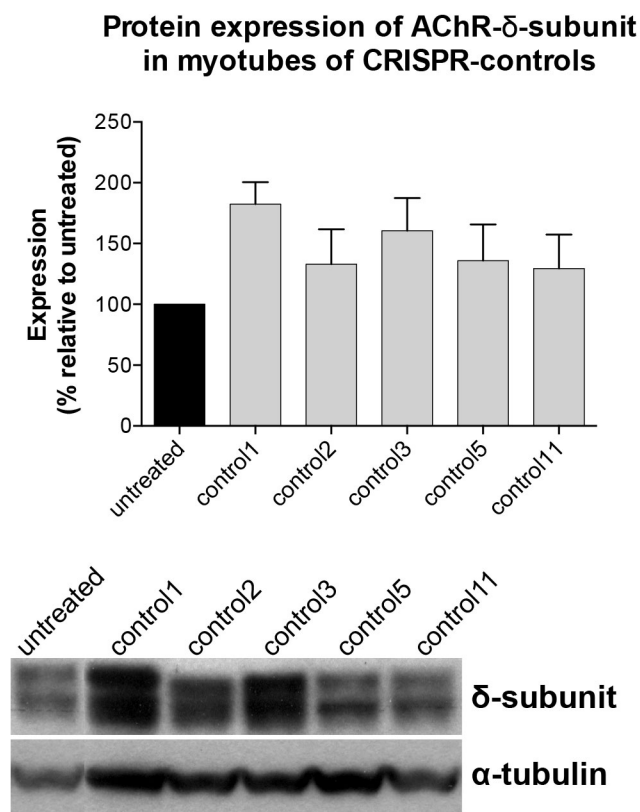


Figure 5-10 Protein expression of AChR δ -subunit in CRISPR-control myotubes

Cell lysates from myotubes were resolved by western blotting analysis using antibodies against δ -subunit and α -tubulin. The results obtained by densitometry analysis (ratio: δ -subunit: α -tubulin), $n=3$, are presented as percentages $\pm$ SEM relative to untreated C2C12. One-way ANOVA and Dunn's multiple comparison test.

No significant difference was detected when the relative ratios of δ -subunit: α -tubulin in control myotubes were compared to untreated myotubes.

5.4.2 The levels of AChR on myotube surfaces of control C2C12 clonal cell lines

An ^{125}I - α -BuTx binding assay was performed on myotubes of the control C2C12 clonal cell lines and untreated C2C12 cells that were either incubated with/without FLaGrin for 16 hours prior to the assay (n=3). Control 3 did not recover its ability to differentiate fully into myotubes and was omitted from this and subsequent analyses. The levels of ^{125}I -BuTx bound on myotube surfaces are presented as percentages relative to untreated C2C12 that were incubated with FLaGrin (Figure 5-11).

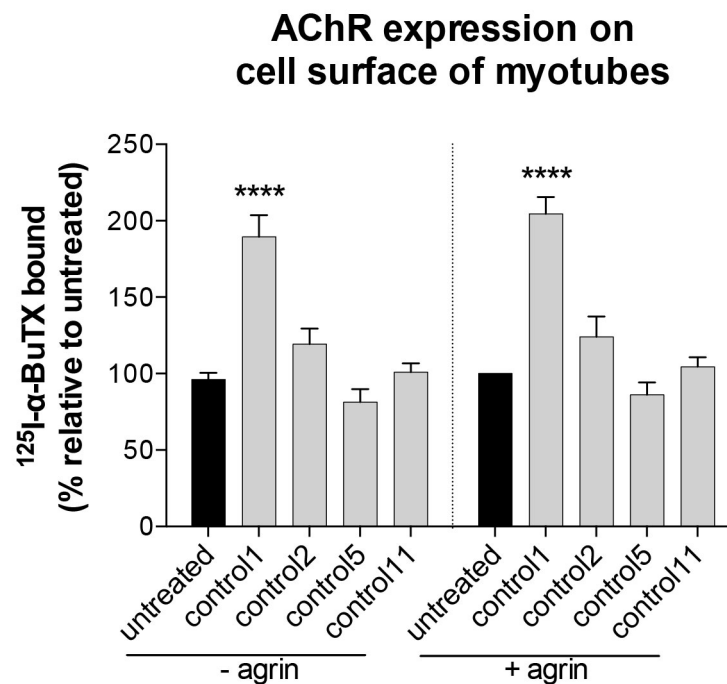


Figure 5-11 ^{125}I - α -BuTx binding to the surfaces of CRISPR-control myotubes

The cell surface expression of AChR on myotubes of untreated C2C12 and CRISPR-control C2C12 clonal cell lines (controls 1, 2, 5 and 11) were measured by the ^{125}I - α -BuTx binding assay. Myotubes were either untreated or treated with FLaGrin for 16 hours prior to the assay.

The results (n=3) were normalised to the levels of ^{125}I - α -BuTx bound on the surfaces of untreated C2C12 that were incubated with FLaGrin. Each bar represents the mean percentages of ^{125}I - α -BuTx bound to myotube surfaces $\pm$ SEM. Two-way ANOVA and Dunn's multiple comparisons test, asterisks ****p<0.0001

Myotubes that were incubated with FLaGrin had a similar amount of ^{125}I -BuTx bound on their surfaces to their respective myotubes that were not stimulated by FLaGrin. When compared to untreated C2C12, significantly higher levels of ^{125}I -BuTx bound on the surfaces of control 1 myotubes were detected for myotubes both untreated and treated with FLaGrin. The relative amount of ^{125}I -BuTx bound on the myotubes of controls 2, 5 and 11 were similar to untreated C2C12.

Control 1 may not be an appropriate control cell line for analysing the pathogenic effects of mutations due to its higher cell surface expression of AChR on myotubes, which may bias the AChR clustering assay. The following investigation into its AChR clustering properties would confirm its suitability for comparison analysis.

5.4.3 Agrin-induced AChR clustering on myotubes of CRISPR-control clonal cell lines of C2C12

The preliminary AChR clustering experiment suggested a possible variation in the AChR clustering properties between CRISPR-control cell lines, therefore the experiment was repeated to quantify the differences between controls 1, 2, 5 and 11 and untreated C2C12 cells. Representative images of the AChR clusters formed are shown in Figure 5-12.

In comparison to untreated C2C12 myotubes, the number and total area of AChR clusters formed on myotubes of controls 1, 2 and 11 were significantly higher. The average area of individual AChR clusters on control 2 was modestly reduced. In contrast, control 5 had significantly reduced total area of AChR clusters, with the average size of AChR clusters reduced to ~50% of the other controls. Although not quantified, AChR clusters formed on control 5 myotubes were visibly dimmer than untreated C2C12 and other control cells (Figure 5-13).

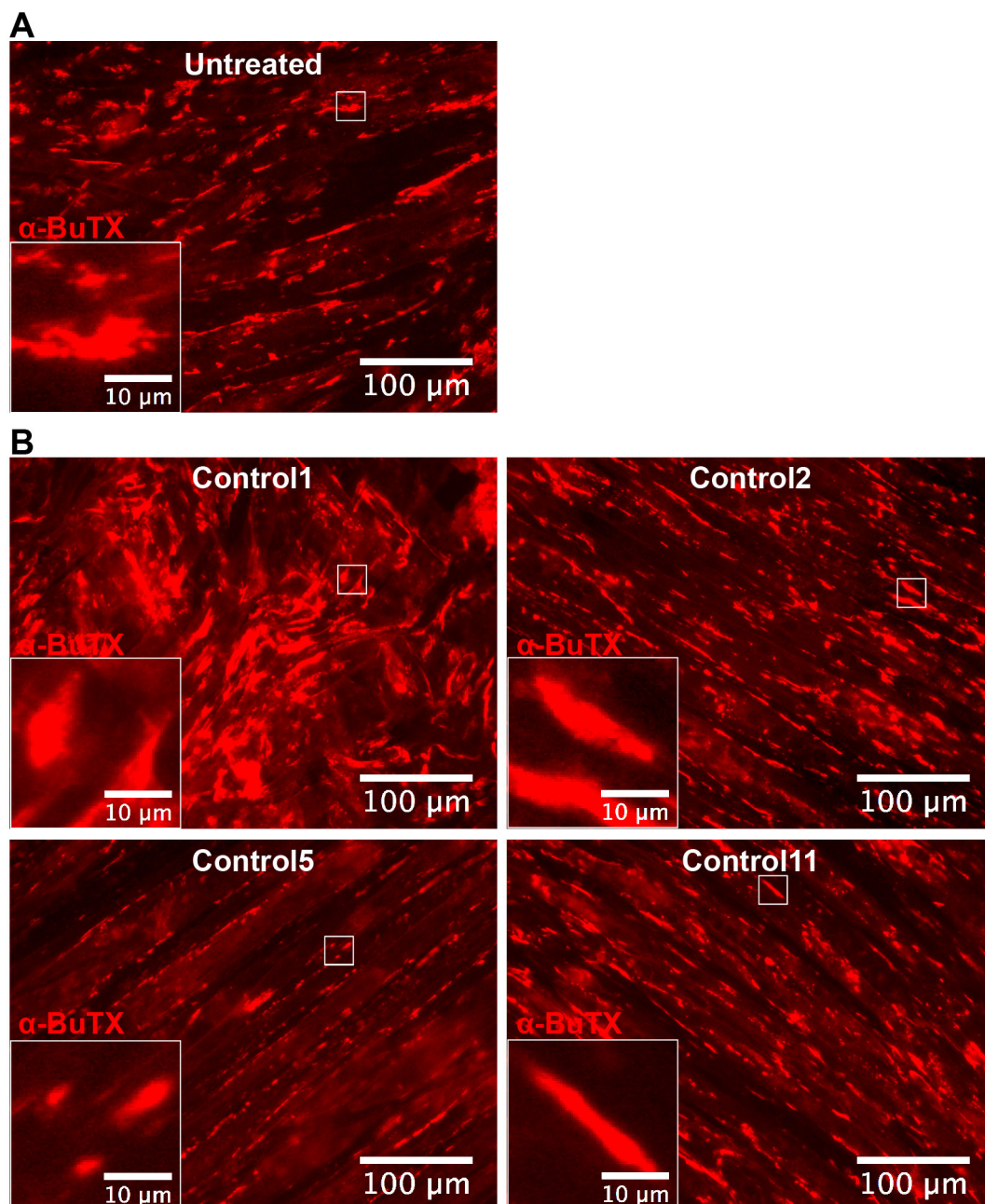
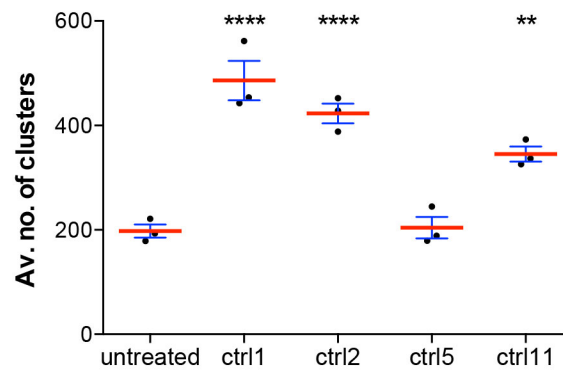


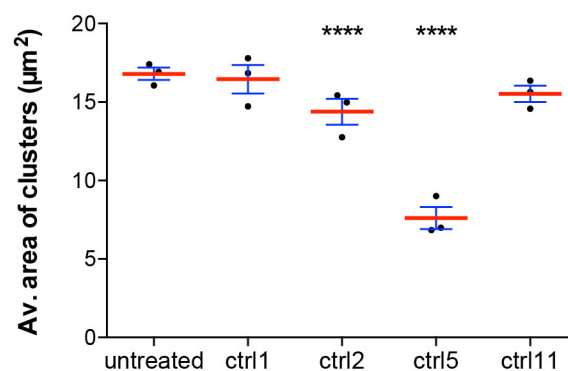
Figure 5-12 Representative images of agrin-induced AChR clustering on myotubes of CRISPR-control cell lines

Untreated C2C12 (**A**) and CRISPR-control clonal cell lines (controls 1, 2, 5 and 11, **B**) were differentiated into myotubes and incubated with FLagrin for 16 hours to induce AChR clustering. The AChR clusters formed were labelled with α-BuTx-594. Each example image represents a microscopic field.

A Number of AChR clusters per field



B Size (area) of AChR clusters



C Total area of clustered AChR per field

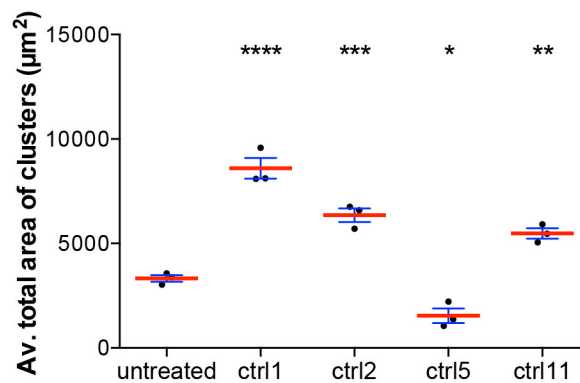


Figure 5-13 Quantified data of AChR clustering on myotubes of CRISPR-control cell lines

The myotubes of untreated C2C12 and CRISPR-control clones 1, 2, 5 and 11 were incubated with 1 in 100 FLaGrin for 16 hours to induce AChR clustering and then labelled with α -BuTx-594. 60 images from 3 experiments were analysed.

Data points represent the mean of data from 20 images for each of the cell lines, and the results are summarised by overall mean $\pm$ SEM. One-way ANOVA and Dunnett's multiple comparisons test, asterisks **** p <0.0001, *** p <0.001, ** p <0.01, * p <0.05

5.4.4 Summary of the properties of the control clonal cell lines of C2C12

Table 5-1 Properties of control clonal cell lines of C2C12

	control1	control2	control3	control5	control11
Differentiation into myotubes	fully	fully	partial	fully	fully
Protein expression of δ -subunit*	=	=	=	=	=
Surface expression of AChR on myotubes*	increased	=	n/a	=	=
Agrin-induced AChR clustering* (number of clusters formed)	increased	increased	n/a	=	increased
Agrin-induced AChR clustering* (average size of clusters formed)	=	reduced	n/a	reduced	=
Agrin-induced AChR clustering* (total area of clusters formed)	increased	increased	n/a	reduced	increased
Morphology of AChR clusters (not quantified)	different	=	n/a	different	=
Conclusion for suitability in further experiments	not suitable	suitable	not suitable	not suitable	suitable

*in comparison to passage-age matched untreated C2C12

After investigations into the protein expression of δ -subunit, the surface expression of AChR and agrin-induced AChR clustering (summarised in Table 5-1), controls 1, 3 and 5 seem to be not suitable for comparison against mutant clones to evaluate the pathogenic properties of the *Chrnd* mutation E381K. Control 1 is the only control clone that had elevated levels, ~2-fold, of cell surface AChR and this may be a contributing cause to the increased AChR clustering compared to untreated C2C12 cells. Control 3 failed to differentiate fully into myotubes, which would hinder analysis. Control 5 myotubes formed agrin-induced AChR clusters that were smaller and dimmer than the untreated C2C12 and other control clones. This was reflected by the reduced average size and total area of the AChR clusters formed. Since data on the properties of AChR clusters, analysed by the Fiji macro, can be influenced by the intensity of the fluorescent signal, control 5 was discarded.

Controls 2 and control 11 were chosen as control cell lines that express δ WT to evaluate the pathogenic properties of the *Chrnd* mutation E381K in subsequent experiments. Although both control 2 and control 11 had a similar protein expression of δ -subunit and surface expression of AChR on myotubes, they gave a significantly higher number and total area of AChR clusters per microscopic field. One possible explanation for the different phenotypes of the control clones could be that the C2C12 were originally a polyclonal population of cells that possess slightly different properties, and this became apparent when the individual clonal cell lines were isolated after the mock CRISPR-Cas9n regime. Another possibility would be the various treatments given to the cells, e.g. electroporation or Geneticin selection, could have been detrimental to the cells and altered their behaviour.

5.5 Establishing C2C12 clonal cell lines that harbour the *Chrnd* mutation E381K

Two different concentrations of CRISPR plasmid (A: 10µg or B: 20µg), that contains the guide oligonucleotide sequences, were co-transfected with the repair template (10µg) into C2C12 myoblasts to identify a suitable concentration to generate mutant cell lines that harbour the *Chrnd* mutation E381K.

Analysis of experiment A: DNA from a total of 71 clones was analysed. From genomic PCR analysis of the region of *Chrnd* comprising the CRISPR target sites, DNA from 49 clones yielded the expected band that is ~750bp in size. *NheI* restriction digest on the PCR products identified 13 candidates that harbour the mutated PAM2 site. Subsequent DNA sequencing on the PCR amplicons from the 13 potential mutant clones has identified a clonal cell line, A11, which is homozygous for the mutation E381K. Both PAM sites of A11 were heterozygously mutated, suggesting HDR with the repair template did not occur for the entire region of the double-stranded DNA breaks generated by Cas9n (Figure 5-14).

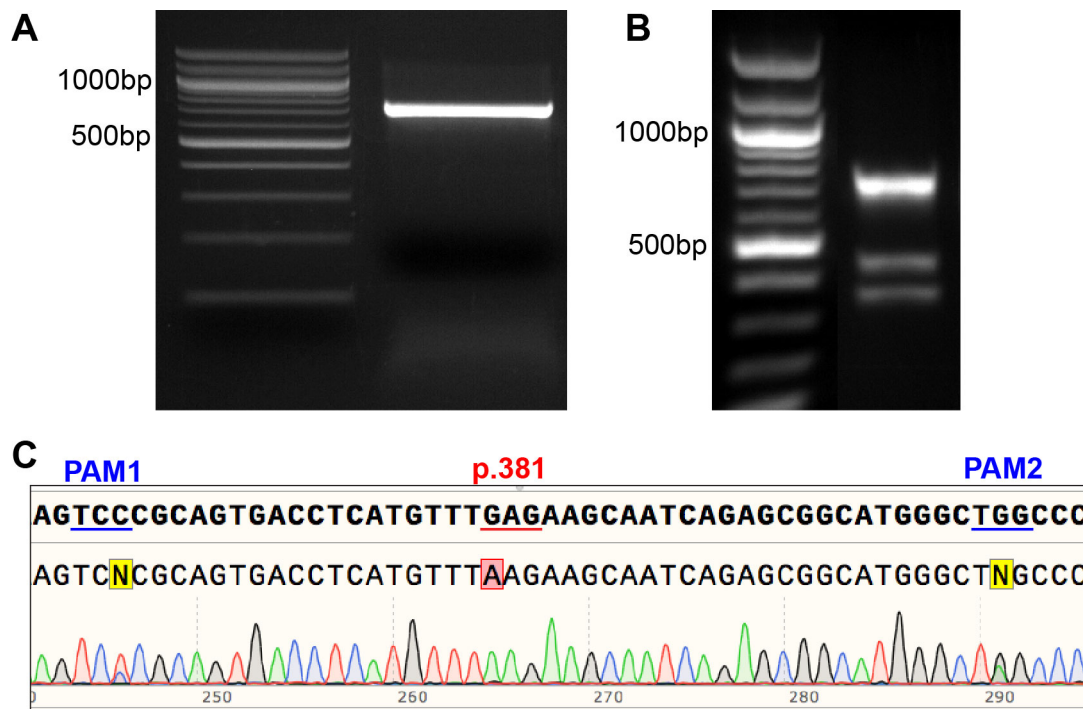


Figure 5-14 Genotype analysis for CRISPR-mutant cell line A11

(A) Genomic PCR analysis of the region of Chrnd comprising the CRISPR target sites for A11 yielded a single band that is ~750bp in size

(B) The restriction enzyme NheI digested the ~750bp product, suggesting the presence of mutated PAM2

(C) DNA sequencing confirmed A11 to be homozygous for the Chrnd mutation E381K, and both PAM sites were heterozygously mutated

Analysis of experiment B: DNA from 49 clones was analysed. The expected single 750bp PCR product was detected for 33 clones, and NheI successfully digested the PCR products of 7 clones. The DNA sequencing results for the PCR amplicon for B15 suggested the presence of E381K, but it was difficult to interpret. To segregate the sequences from the 2 different alleles, the PCR products were subcloned into expression plasmids and transformed into JM-109 competent cells. The sequencing of 6 purified plasmids identified 2 different sequences, and confirmed B15 to be heterozygous for the mutation E381K with a 13bp deletion that overlaps with the E381 loci. (Figure 5-15)

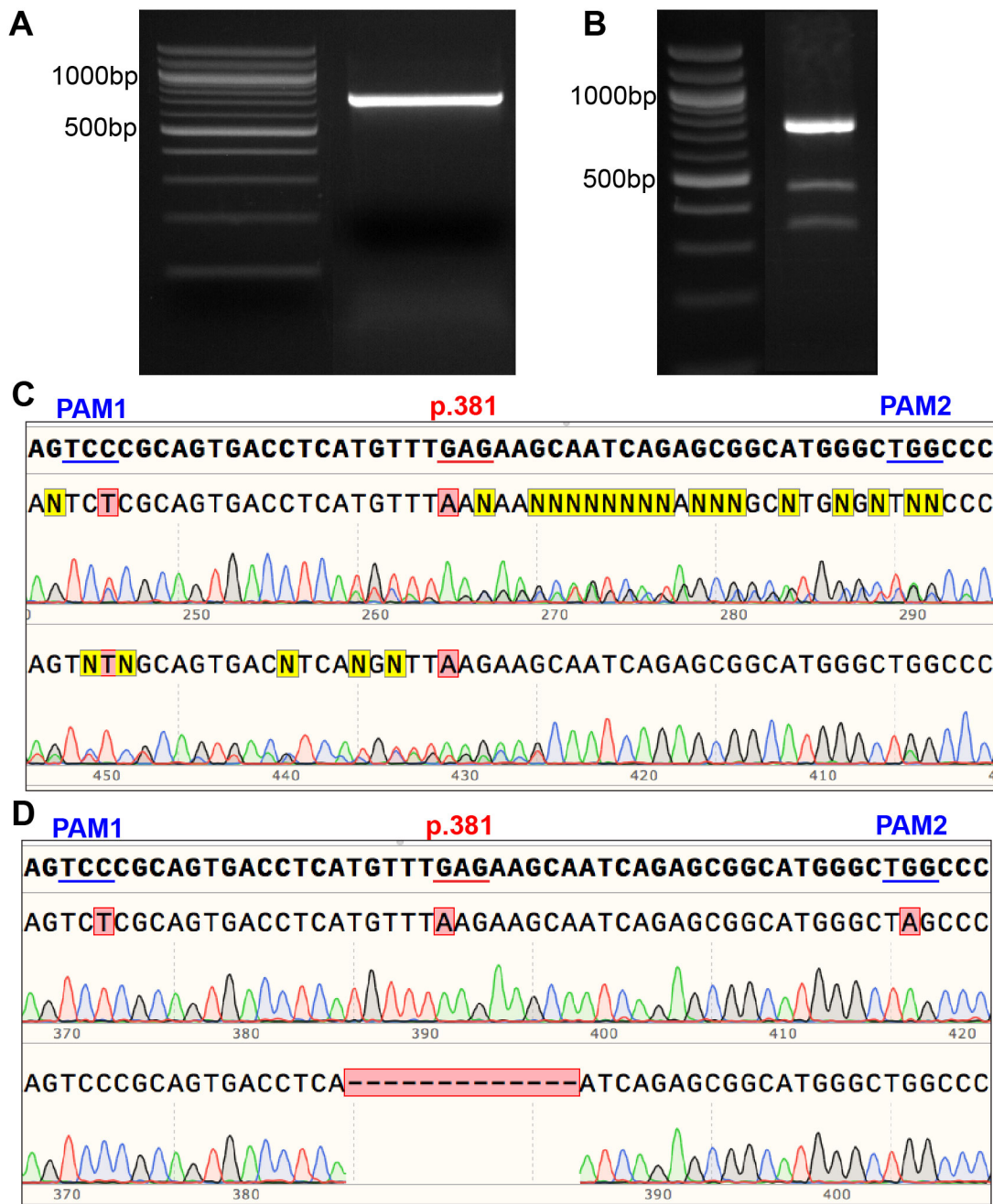


Figure 5-15 Genotype analysis for CRISPR-mutant cell line B15

(A) Genomic PCR analysis of the region of *Chrnd* comprising the CRISPR target sites for B15 yielded a single band that is ~750bp in size

(B) The restriction enzyme *NheI* digested the ~750bp product, suggesting the presence of mutated PAM2

(C-D) Initial DNA sequencing results suggested the presence of the mutation GAG>AAG for E381K, but this was difficult to interpret. Hence the PCR products were subcloned into expression plasmids and segregated. Sequencing on 6 purified plasmids confirmed B15 to be heterozygous for the mutation E381K with a 13bp deletion, c.del1136_1148TGTTTGAGAAGCA, which overlaps with the E381 loci

The 13bp mutation, c.del1136_1148TGTTTGAGAAGCA, in B15 leads to a frameshift to the coding sequence for 31 amino acids, followed by a premature stop codon (Figure 5-16 A). This results in the loss of the entire M4 transmembrane domain of the δ -subunit of AChR, and the allele should therefore be non-functional.

To determine if the mutant c.del1136_1148TGTTTGAGAAGCA δ -subunit forms AChR that are expressed on the cell membrane surface, cDNA encoding mouse c.del1136_1148TGTTTGAGAAGCA- or WT- δ -subunits of AChR were transfected with mouse WT- α -, β -, ϵ - subunits, at a ratio of 2 α :1 β :1 δ :1 ϵ , together with the transfection marker EGFP into HEK 293 cells. Forty-eight hours after transfection, successful transfection was confirmed by fluorescence microscopy and cell surface expression of AChR was quantified by the ^{125}I - α -BuTx binding assay.

The levels of ^{125}I - α -BuTx bound on untransfected or mock transfected with EGFP HEK 293 cells were minimal. When compared to cells transfected to express WT-AChR, the relative level of ^{125}I - α -BuTx bound on cells expressing AChR with the mutant δ -subunit was reduced to the background level. As expected, the c.del1136_1148TGTTTGAGAAGCA deletion in *Chrnd* ablates the formation of functional AChR (Figure 5-16 B).

The clonal cell line B15 would allow investigations into the functional effects of δ E381K without masking the effects from its second allele. The patient that was originally reported is also heterozygous for the *CHRND* mutation E381K together with a non-functional second allele due to a 2.2kb chromosomal deletion (Muller et al., 2006b). Thus B15 may mimic the cellular phenotype of the affected patient.

A

1	LNEEQRLIQHLFNEKGYDKDLRPVARKEDKVDVALSLTSLNLSLKEVEETLTNTNVWDH	60
1	LNEEQRLIQHLFNEKGYDKDLRPVARKEDKVDVALSLTSLNLSLKEVEETLTNTNVWDH	60
61	AWVDSRLQWDANDFGNITVLRLLPDMVWLPEIVLENNNDGSFQISYACNVLVYDSGYVTW	120
61	AWVDSRLQWDANDFGNITVLRLLPDMVWLPEIVLENNNDGSFQISYACNVLVYDSGYVTW	120
121	LPPAIFRSSCPISVTYFPFDWQNCSLKFSSLYTAKEITLSLKQEEENNRSYPIEWIID	180
121	LPPAIFRSSCPISVTYFPFDWQNCSLKFSSLYTAKEITLSLKQEEENNRSYPIEWIID	180
181	PEGFTENGWEIVHRAAKLNVDPSVPMDSSTNHQDVTFYLIIRRKPLFYIINILVPCVLIS	240
181	PEGFTENGWEIVHRAAKLNVDPSVPMDSSTNHQDVTFYLIIRRKPLFYIINILVPCVLIS	240
241	FMINLVFYLPGDCGEKTSVAISVLLAQSVFLLISKRLPATSMAPLVGKFLFGLFVLT	300
241	FMINLVFYLPGDCGEKTSVAISVLLAQSVFLLISKRLPATSMAPLVGKFLFGLFVLT	300
301	MVVVICVIVLNIHFRTPTSTHVLSEGVKKFFLETLPKLLHMSRPAEEDPGPRALIRRSSL	360
301	MVVVICVIVLNIHFRTPTSTHVLSEGVKKFFLETLPKLLHMSRPAEEDPGPRALIRRSSL	360
361	GYICKAEYFSLKSRSDLMFEKQSERHGLARRLTTARRPPASSEQVQQLFNMKPAVDG	420
361	GYICKAEYFSLKSRSDLNQSGMGWGPAGLQQAALSRSSMR	409
421	ANFIVNHMRDQNSYNEEKDNWNQVARTVDRLCLFVVTVMVVGTAWIFLQGVYNQPLQP	480
481	FPGDPFSYSEQDKRFI	496

WT
 c.del1136_1148TGTTTGAGAAGCA

B

**AChR expression on
cell surface of transfected HEK 293 cells**

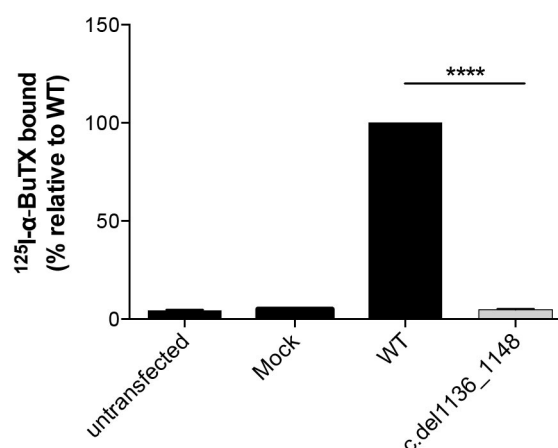


Figure 5-16 Surface expression of AChR assembled with the c.del1136_1148TGTTTGAGAAGCA mutant δ -subunit

(A) Alignment of amino acid sequences (minus signal peptide) of WT- and mutant- δ -subunit of AChR. The 13bp mutation leads to a frameshift in the coding sequence of *Chrmd* (highlighted in grey), followed by a premature stop codon.

(B) ¹²⁵I- α -BuTx binding to the surface of HEK 293 cells. WT- or c.del1136_1148TGTTTGAGAAGCA mutant- δ subunits were transfected with WT- α -, β - and ϵ -subunits into cells at a ratio of 2 α :1 β :1 δ :1 ϵ by PEI transfection method. EGFP was cotransfected with AChR as a transfection efficiency marker and successful transfection was confirmed by fluorescence microscopy. Mock = cells transfected with EGFP only. The results (n=3) are presented as mean percentages $\pm$ SEM relative to cells expressing WT-AChR. Unpaired t test, asterisks ****p<0.0001.

5.6 Analysis of δ E381K using clonal cell lines generated by CRISPR-Cas9n mutagenesis

Four clonal cell lines were generated from C2C12 myoblasts to investigate the functional effects of the *Chrnd* mutation, E381K. Controls 2 and 11 represent cells that express δ WT; A11 is homozygous for E381K δ and B15 is heterozygous for E381K δ with a truncating deletion on its second allele, which was confirmed as non-functional. Untreated C2C12 cells were also included as an additional control.

5.6.1 The effect of the *Chrnd* E381K mutation on the protein expression of AChR δ -subunit

The expression levels of AChR subunits can impact on AChR assembly, which may affect AChR clustering. To confirm the underlying mechanisms of δ E381K, the expression of δ -subunit in myotubes of untreated C2C12, control 2, control 11, A11 and B15 was analysed first. Whole cell lysates were prepared from myotubes and were resolved by western blotting with antibodies against the δ -subunit and α -tubulin. The ratio of δ -subunit: α -tubulin (n=3) was obtained by densitometry analysis and the results were normalised to the expression levels of control 2 (Figure 5-17 A).

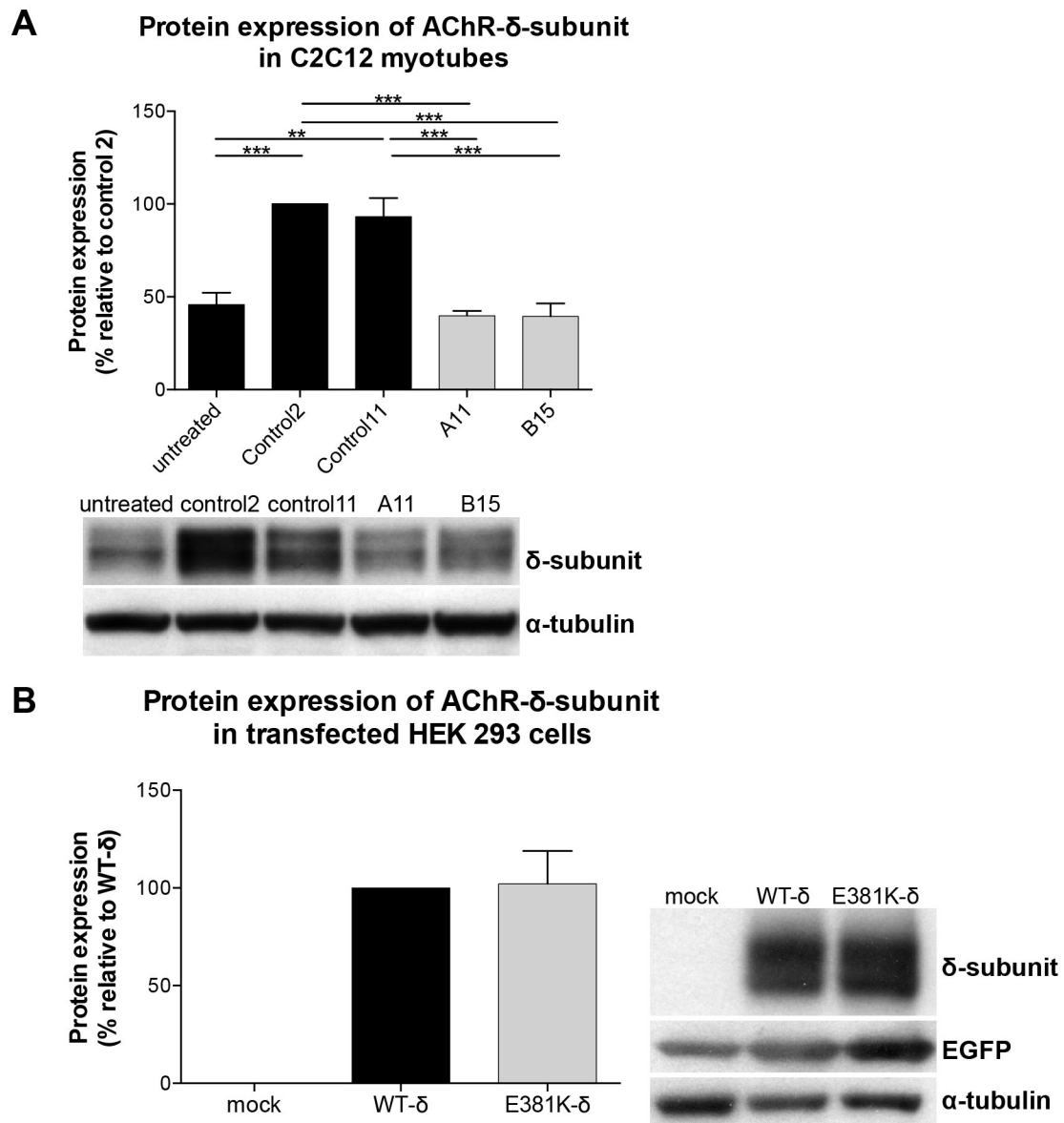


Figure 5-17 The protein expression of the δ E381K in A11/B15 mutant C2C12 myotubes and transfected HEK 293 cells

(A) Cell lysates from untreated C2C12, CRISPR-control (controls 2 and 11) and δ E381K mutant (A11, B15) myotubes were resolved by western blotting analysis using antibodies against δ -subunit and α -tubulin. **(B)** To confirm the functional effects of δ E381K, AChR with WT- or E381K- δ -subunits were overexpressed in HEK 293 cells with the subunit ratio of 2 α :1 β :1 δ :1 ϵ . Cell lysates were prepared 48 hours after transfection and resolved as described.

Results obtained by densitometry analysis (ratio: δ -subunit: α -tubulin), $n=3$, are presented as percentages $\pm$ SEM relative to (A) control 2 and (B) WT-AChR. One-way ANOVA and Tukey's multiple comparisons test were used for (A), and unpaired t test for (B), asterisks *** $p<0.001$, ** $p<0.01$

Similar expression levels of δ -subunit were detected from untreated C2C12, A11 and B15 myotubes. Conversely, A11 and B15 myotubes had significantly reduced amounts of δ -subunit when compared to controls 2 and 11.

The levels of δ -subunit in myotubes of controls 2 and 11 were twice as high compared to untreated C2C12. The expression levels of AChR subunits increases during the process of differentiation into myotubes (Sanes and Lichtman, 2001). Hence in addition to the semi-quantitative nature of western blotting, variations in the abilities of the C2C12 cells to differentiate into myotubes suggest that it is technically challenging to reliably determine the effects of mutations on the protein levels of AChR subunits in C2C12 cells.

To confirm the effect of the mutation on the protein levels of the δ -subunit clearly, δ WT- or δ E381K- AChR were overexpressed in HEK 293 cells at the ratio of $2\alpha:1\beta:1\delta:1\varepsilon$ to obtain further results (n=3) (Figure 5-17 **B**).

The expression levels of δ -subunits in HEK cells expressing AChR with mutant E381K- δ -subunits were found to be indifferent to cells expressing WT-AChR. Thus the E381K mutation does not affect the protein expression of the δ -subunit.

5.6.2 Cell surface expression of AChR on A11 and B15 mutant cells

The level of AChR assembled with human δ E381K, when overexpressed in HEK 293 cells, on cell surface was found to be ~70% of the WT control (Muller et al., 2006b). The ^{125}I - α -BuTx binding assay was performed on the myoblasts and myotubes of untreated C2C12 and CRISPR-generated control and mutant cells. Myotubes were either untreated or stimulated by FLaGrin for 16 hours to evaluate if AChR clustering alters the expression of cell surface AChR. The results quantified by ^{125}I - α -BuTx bound on cell surface are presented as percentages relative to the expression of control 2 for myoblasts and control 2 that were incubated with FLaGrin for myotubes.

The levels of ^{125}I - α -BuTx bound on the cell surfaces of controls 2 and 11 and untreated C2C12 myoblasts were similar, which agrees with the previous analysis of control clonal cell lines. In comparison with the controls, significantly reduced amount of ^{125}I - α -BuTx bound was detected from the myoblasts of A11 and B15 (Figure 5-18 A).

Similarly, significantly less ^{125}I - α -BuTx was bound to A11 and B15 myotubes when compared to the controls. Incubation of myotubes with FLaGrin prior to the assay, i.e. AChR clustering did not alter the relative steady-state level of ^{125}I - α -BuTx bound on cell surfaces (Figure 5-18 B).

The results are therefore in agreement with published results that showed reduction in the cell surface expression of AChR caused by the mutation (Muller et al., 2006b).

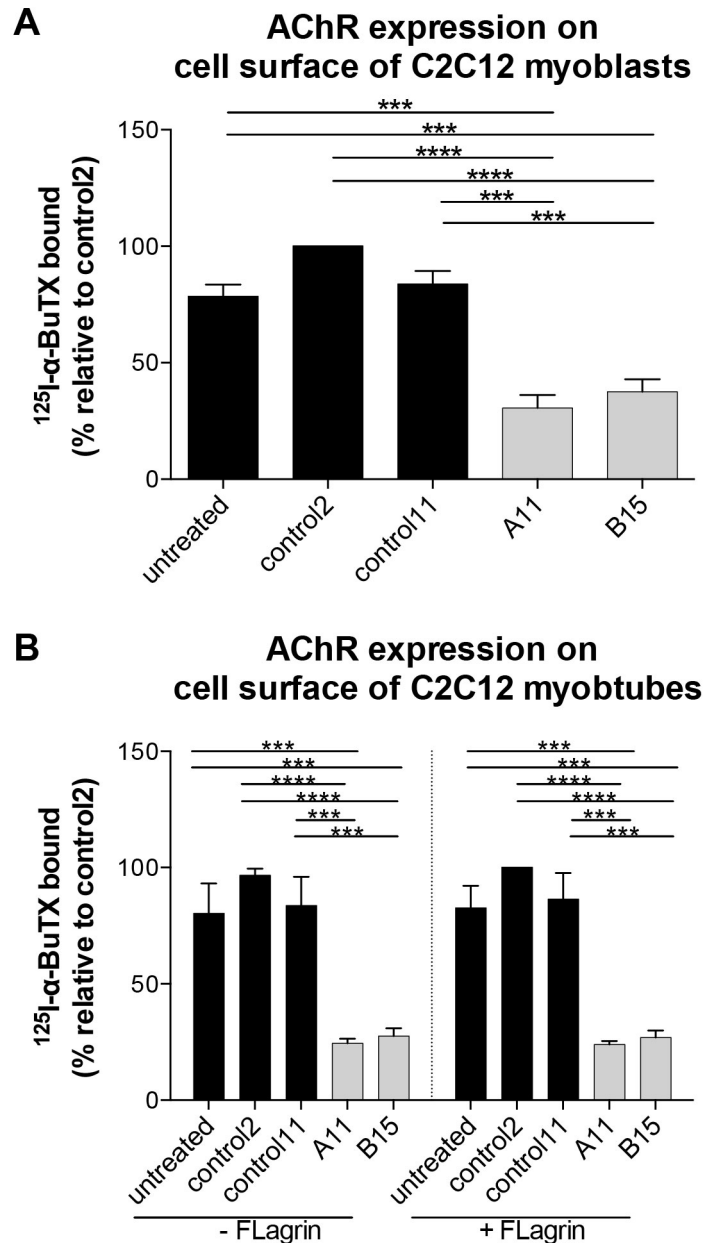


Figure 5-18 ¹²⁵I-α-BuTx binding to the surfaces of CRISPR- control and δE381K mutant cells

The cell surface expression of AChR on δE381K mutant **(A)** myoblasts and **(B)** myotubes, A11 and B15, were measured by the ¹²⁵I-α-BuTx binding assay and compared to untreated C2C12 and CRISPR-control cells, controls 2 and 11. Myotubes were either untreated or incubated with FLaigrin for 16 hours prior to the assay.

The results (n=3) were normalised to the levels of ¹²⁵I-α-BuTx bound on the surfaces of **(A)** control 2, and **(B)** control 2 incubated with FLaigrin. Each bar represents the mean percentages of ¹²⁵I-α-BuTx bound to myotube surfaces ±SEM. **(A)** One-way ANOVA and Tukey's multiple comparisons tests; **(B)** Two-way ANOVA and Sidak's multiple comparisons test, asterisks ****p<0.0001, ***P<0.001.

5.6.3 Agrin-induced AChR clustering on A11 and B15 mutant myotubes

The agrin-induced AChR clustering properties of the δ E381K mutant cells, A11 and B15, were investigated by incubating myotubes with 1 in 100 FLagrin or 1 in 10 FLagrin for 16 hours prior to labelling of AChR with α -BuTx-594. Examples of the microscopic fields the AChR clusters formed are shown in Figure 5-19 and Figure 5-20. The quantified results are shown in Figure 5-21.

There are variations in the AChR clustering properties between control cells. Supporting the previous findings, more AChR clusters were formed on control 2 and 11 myotubes when compared to untreated C2C12. This resulted in larger total areas of clustered AChR in control 2 and control 11 myotubes.

The numbers, average sizes and total areas of AChR clusters formed on δ E381K mutant A11 and B15 myotubes were significantly reduced when compared to the controls, except when the number of clusters induced by 1 in 100 FLagrin on untreated and B15 were compared.

The pattern of results between AChR clusters induced by 1 in 100FLagrin and 1 in 10FLagrin were consistent.

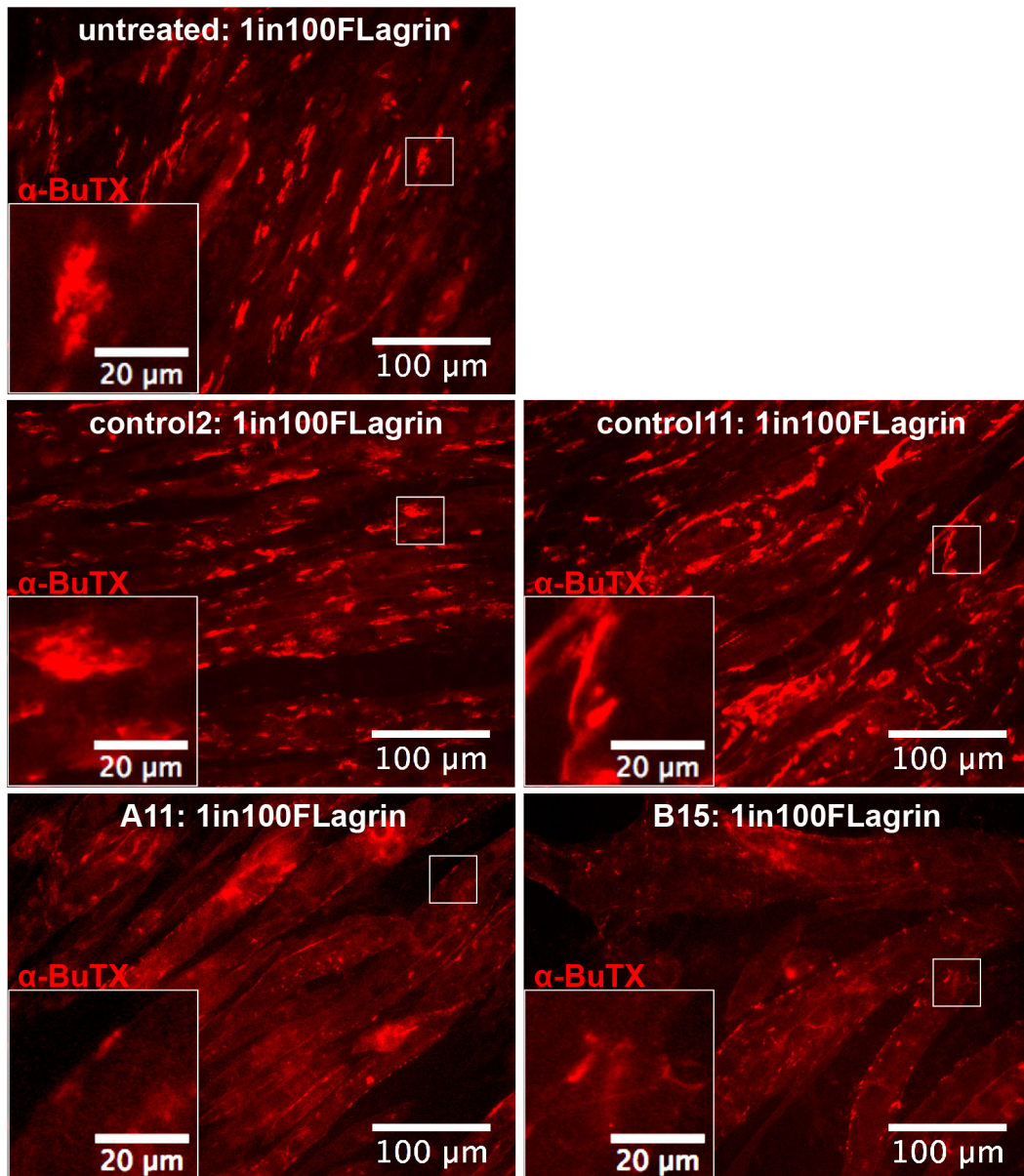


Figure 5-19 Representative images of 1 in 100FLagrin-induced AChR clustering on control and δ E381K cell lines

Control (untreated C2C12, control 2 and control 11) and δ E381K-mutant (A11 and B15) C2C12 cells were differentiated into myotubes and incubated with 1 in 100FLagrin for 16 hours to induce AChR clustering. AChR clusters formed were labelled with α -BuTx-594. Each example image represents a microscopic field.

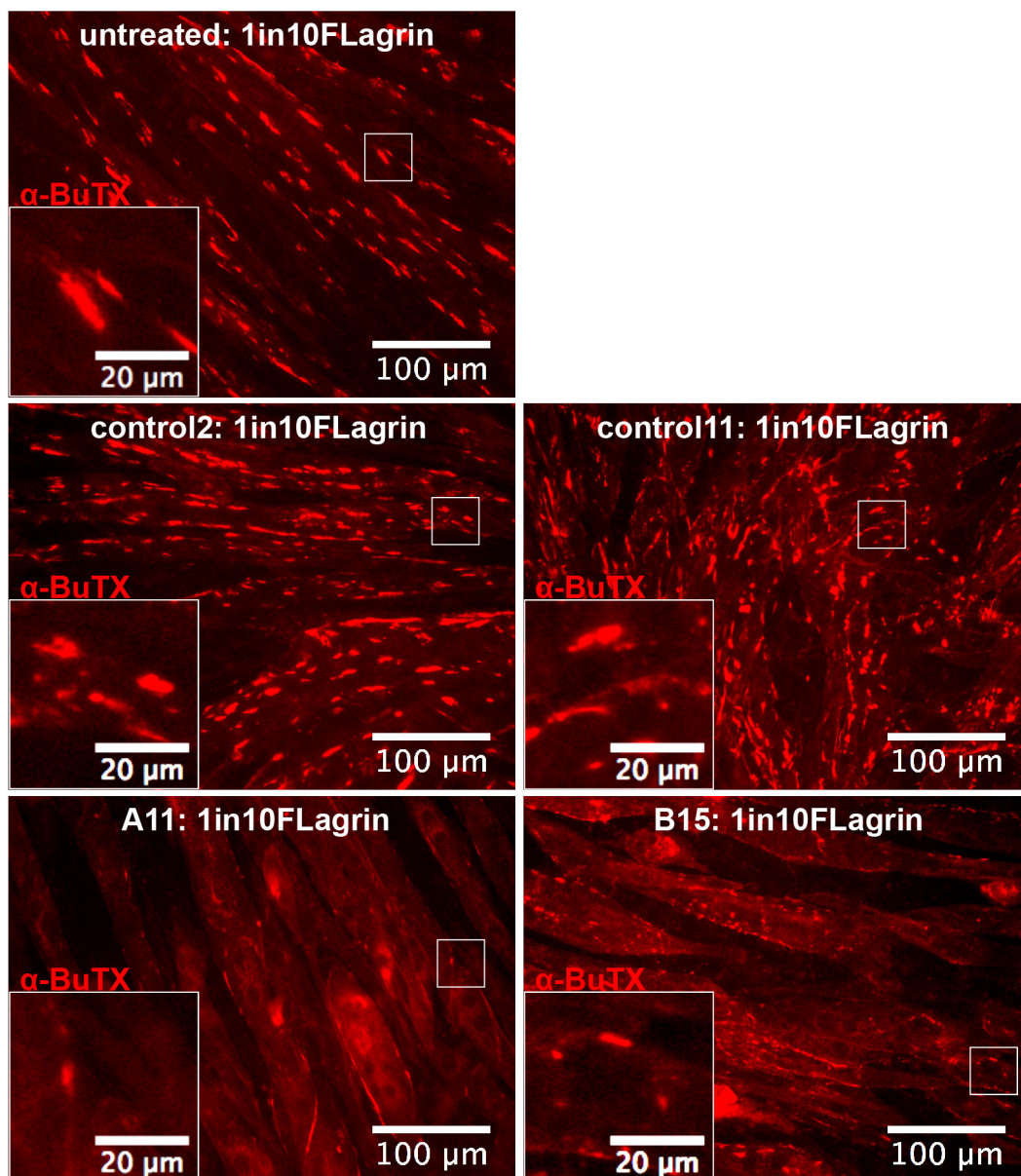


Figure 5-20 Representative images of 1 in 10FLagrin-induced AChR clustering on control and δ E381K cell lines

Control (untreated C2C12, control 2 and control 11) and δ E381K-mutant (A11 and B15) C2C12 cells were differentiated into myotubes and incubated with 1 in 10 FLagrin for 16 hours to induce AChR clustering. AChR clusters formed were labelled with α -BuTx-594. Each example images represent a microscopic field.

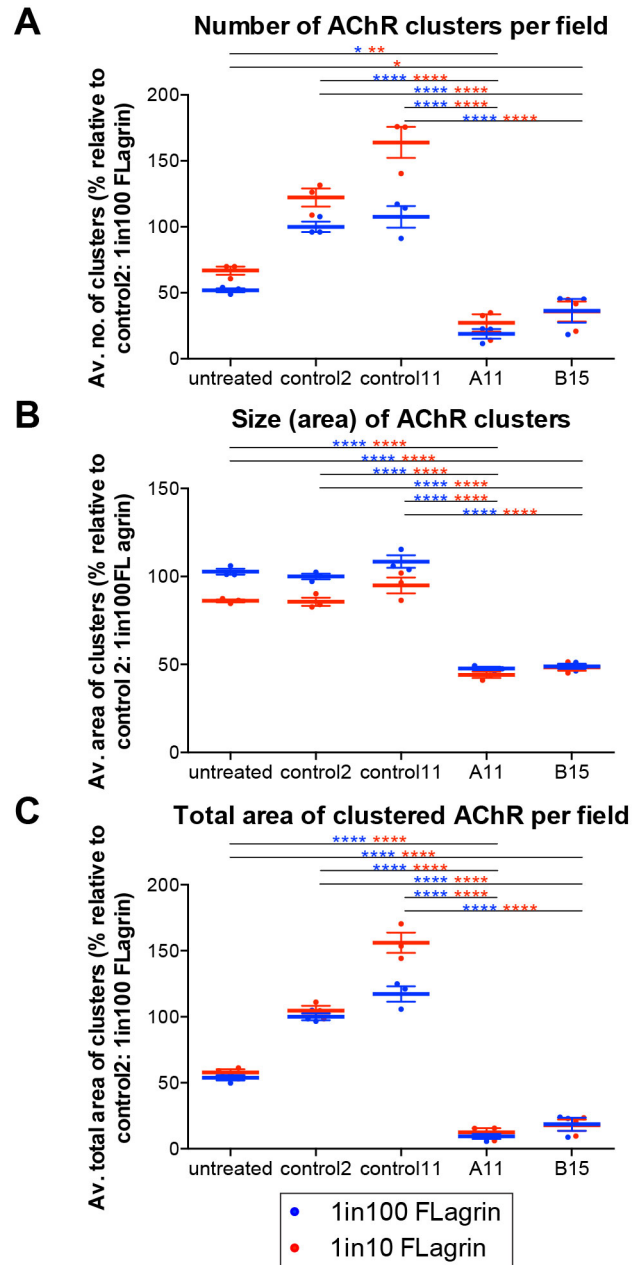


Figure 5-21 Quantified data on agrin-induced AChR clustering on CRISPR-control and δ E381K cell lines

Control (untreated C2C12, control 2 and control 11) and δ E381K (A11 and B15) myoblasts were differentiated into myotubes and incubated with either 1in100FLagrin (blue) or 1 in 10 FLaGrin (red) for 16 hours to induce AChR clustering. AChR clusters formed were labelled with α -BuTx-594 and quantified.

60 images from 3 experiments were analysed for each cell line. Each data point represents the mean from 20 images, and the results are normalised to control 2 (mean percentage $\pm$ SEM). One-way ANOVA and Tukey's multiple comparisons test, asterisks **** p <0.0001; ** p <0.01, * p <0.05.

5.7 Generation of *Chrnd*-knockout cells using the CRISPR-Cas9n system

The previous section has demonstrated that the investigation into the functional effect of δ E381K was made difficult due to the variations between control C2C12 clonal cell lines. A second way to look at the functional effects is to introduce δ WT or δ E381K into *Chrnd*-knockout (KO) cells, which could eliminate many of the variables. *Chrnd*-KO cell lines were generated using the CRISPR-Cas9n system.

To introduce loss-of-function mutations in the *Chrnd* of C2C12 cells, 10 μ g of the CRISPR plasmid containing the guides A and B was electroporated into C2C12 myoblasts without the repair template to promote spontaneous indels that would cause a frameshift in the δ -subunit transcript. Myoblasts of 12 clones were differentiated into myotubes for agrin-induced AChR clustering to preliminarily identify clones that do not form AChR clusters. Nine clones were able to completely differentiate into myotubes, 2 of which did not form AChR clusters after incubation with 1 in 100FLagrin. Clonal cell line KO6 was found to readily differentiate into myotubes and was chosen for further experiments. Sequencing on the genomic PCR product of KO6 (a single band at ~750bp on agarose electrophoresis) identified 3 variants for *Chrnd*: 2 different 13bp deletions c.del1136_1148TGTTTGAGAAGCA (c.del1136_1148) and c.del1131_1143CCTCATGTTTGAG (c.del1131_1143), and a 25bp deletion c.del1132_1156CTCATGTTTGAGAAGCAATCAGAGC (c.del1132_1156) (Figure 5-22).

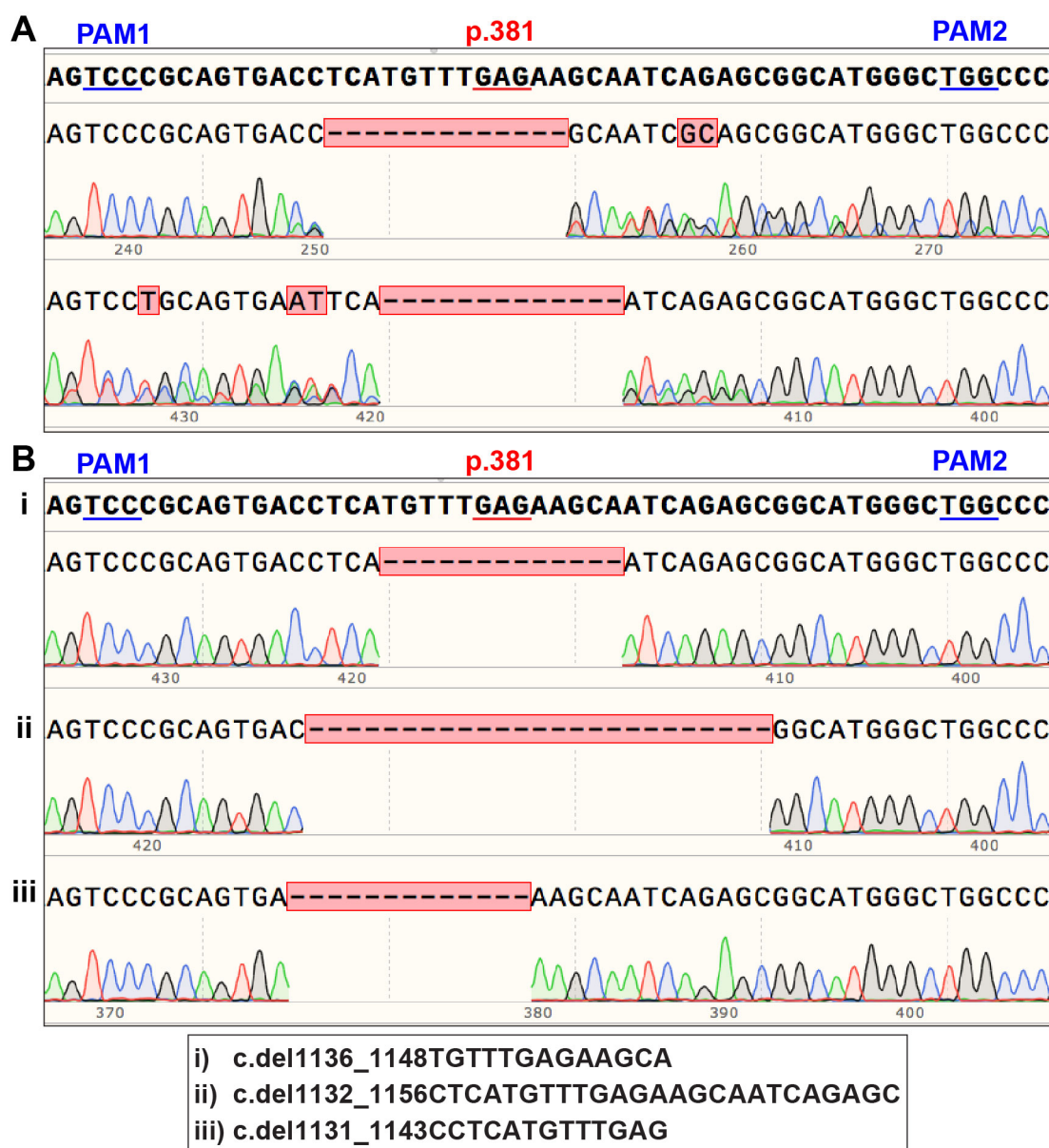


Figure 5-22 Genotype analysis for CRISPR-mutant cell line KO6

(A) Initial DNA sequencing on the genomic PCR amplicon that includes the region of the CRISPR target sites suggested the presence of deleting mutations in proximity to the p.381 loci of the *Chrnd* of the clonal cell line KO6

(B) The PCR products from the genomic DNA of KO6 were subsequently subcloned into expression plasmids, pGEM-T-Easy and segregated. Ten of the purified plasmids were analysed by DNA sequencing and 3 different variants of *Chrnd* were identified:

i) 13bp deletion c.del1136_1148TGTTTGAGAAGCA

ii) 25bp deletion c.del1132_1156CTCATGTTTGAGAAGCAATCAGAGC

iii) 13bp deletion c.del1131_1143CCTCATGTTTGAG

There are 2 possible reasons for 3 different variants of *Chrnd* to be identified from the cell line KO6. Colleagues in our laboratory have also found more than 2 alleles in other C2C12 clonal cells (unpublished observations), suggesting that C2C12 is very likely to be a polyploidy cell line, and more than 2 copies of the *Chrnd* are present in a single cell. A study that characterised the morphology of C2C12 cells during the differentiation process has identified some ‘giant cells’ that were suggested to possess a very large polyploid nucleus (Burattini et al., 2004). Although only 2 variants of *Chrnd* were identified from the A11 and B15 mutant clones, in that experiment, the screening procedure eliminated clones that do not possess the PAM2 mutation indicated by restriction digestion. Therefore the possibility of the presence of polyploid cells should not be excluded. Another possibility could be the generation of a new indel after the clonal isolation within the KO6 cells, as the action of Cas9n to nick DNA at the target sites would remain until the residual CRISPR plasmids were degraded or diluted through cell division.

The *Chrnd* variant c.del1136_1148 is also present in the B15 clone, and previous analysis confirmed that this mutation completely prevents the expression of AChR on cell surface. Similar to c.del1136_1148, the mutations c.del1131_1143 and c.del1132_1156 cause a frameshift and lead to 28 and 33 missense amino acids respectively, followed by a premature stop codon (Figure 5-23). Hence all of the variants identified from the genomic DNA of KO6 lead to the loss of the entire M4 transmembrane domain, and will result in a non-functional δ -subunit. Since the objective of this work was to generate a mutant cell line of C2C12 that does not express functional δ -subunit of AChR, therefore the presence of 3 alleles within the KO6 cells should not matter.

1	LNEEQRLIQHLFNEKGYDKDLRPVARKEDKVDVALSLTSLNLI	SLKEVEETLT	TNNVWIDH	60	
1	LNEEQRLIQHLFNEKGYDKDLRPVARKEDKVDVALSLTSLNLI	SLKEVEETLT	TNNVWIDH	60	
1	LNEEQRLIQHLFNEKGYDKDLRPVARKEDKVDVALSLTSLNLI	SLKEVEETLT	TNNVWIDH	60	
1	LNEEQRLIQHLFNEKGYDKDLRPVARKEDKVDVALSLTSLNLI	SLKEVEETLT	TNNVWIDH	60	
61	AWVDSRLQWDANDFGNITVLRLPPDMVWLPEIVLENNNDGSFQISYACNVLVYDSGYVTW			120	
61	AWVDSRLQWDANDFGNITVLRLPPDMVWLPEIVLENNNDGSFQISYACNVLVYDSGYVTW			120	
61	AWVDSRLQWDANDFGNITVLRLPPDMVWLPEIVLENNNDGSFQISYACNVLVYDSGYVTW			120	
61	AWVDSRLQWDANDFGNITVLRLPPDMVWLPEIVLENNNDGSFQISYACNVLVYDSGYVTW			120	
121	LPPAIFRSSCPISVTYFFPDWQNC	SLKFSS	LKYTAKEITLSLKQEEENNR	SYPIEWIID	180
121	LPPAIFRSSCPISVTYFFPDWQNC	SLKFSS	LKYTAKEITLSLKQEEENNR	SYPIEWIID	180
121	LPPAIFRSSCPISVTYFFPDWQNC	SLKFSS	LKYTAKEITLSLKQEEENNR	SYPIEWIID	180
121	LPPAIFRSSCPISVTYFFPDWQNC	SLKFSS	LKYTAKEITLSLKQEEENNR	SYPIEWIID	180
181	PEGFTENGWEIVHRAAKLNVDPSVPMDS	TNHQDVTFYLI	IRRKPLFYI	INILVPCVLIS	240
181	PEGFTENGWEIVHRAAKLNVDPSVPMDS	TNHQDVTFYLI	IRRKPLFYI	INILVPCVLIS	240
181	PEGFTENGWEIVHRAAKLNVDPSVPMDS	TNHQDVTFYLI	IRRKPLFYI	INILVPCVLIS	240
181	PEGFTENGWEIVHRAAKLNVDPSVPMDS	TNHQDVTFYLI	IRRKPLFYI	INILVPCVLIS	240
241	FMINLVFYLPGDCGEKTSVAISVLLAQSVFLL	LISKRLPAT	SMAIPLVGK	FLLFGMVLVT	300
241	FMINLVFYLPGDCGEKTSVAISVLLAQSVFLL	LISKRLPAT	SMAIPLVGK	FLLFGMVLVT	300
241	FMINLVFYLPGDCGEKTSVAISVLLAQSVFLL	LISKRLPAT	SMAIPLVGK	FLLFGMVLVT	300
241	FMINLVFYLPGDCGEKTSVAISVLLAQSVFLL	LISKRLPAT	SMAIPLVGK	FLLFGMVLVT	300
301	MVVVICVIVLNIHFRTPTSTHVLSEGVKKFFLETLPKLLHMSRPAEEDPGPRALIRRSSSL			360	
301	MVVVICVIVLNIHFRTPTSTHVLSEGVKKFFLETLPKLLHMSRPAEEDPGPRALIRRSSSL			360	
301	MVVVICVIVLNIHFRTPTSTHVLSEGVKKFFLETLPKLLHMSRPAEEDPGPRALIRRSSSL			360	
301	MVVVICVIVLNIHFRTPTSTHVLSEGVKKFFLETLPKLLHMSRPAEEDPGPRALIRRSSSL			360	
361	GYICKAEYFSLKSRSDLN	QSGMGWPGAS	PQAGLQQALSR	SNRSSMR	409
361	GYICKAEYFSLKSRSDLN	QSGMGWPGAS	PQAGLQQALSR	SNRSSMR	409
361	GYICKAEYFSLKSRSD	GMWPGAS	PQAGLQQALSR	SNRSSMR	405
361	GYICKAEYFSLKSRSE	SNQSGMGWPGAS	PQAGLQQALSR	SNRSSMR	409
421	ANFIVNHMRDQNSYNEEKDNWNQVARTVDRLCLFVVTPVMVGTAWIFLQGVYNQPPLQP			480	
481	FPGDPFSYSEQDKRFI			496	

WT

c.del1136_1148TGTTTGAGAAGCA

c.del1132_1156CTCATGTTTGAGAAGCAATCAGAGC

c.del1131_1143CCTCATGTTTGAG

Figure 5-23 Alignment of amino acid sequences (minus signal peptide) of mutant-*Chrnd* alleles in KO6 to WT sequences

The mutations identified from variants of the δ -subunits in KO6, c.del1136_1148TGTTTGAGAAGCA (blue), c.del1132_1156CTCATGTTTGAGAAGCAATCAGAGC (red) and c.del1131_1143CCTCATGTTTGAG (green) shift the coding sequences of *Chrnd* (highlighted in grey) and result in the truncations of the δ -subunit proteins.

To confirm that the detected deletions in *Chrnd* prevent the cell surface expression of AChR, WT- or mutant- AChR were overexpressed in HEK 293 cells, and the expressions of AChR on cell surfaces were measured by ^{125}I - α -BuTx binding.

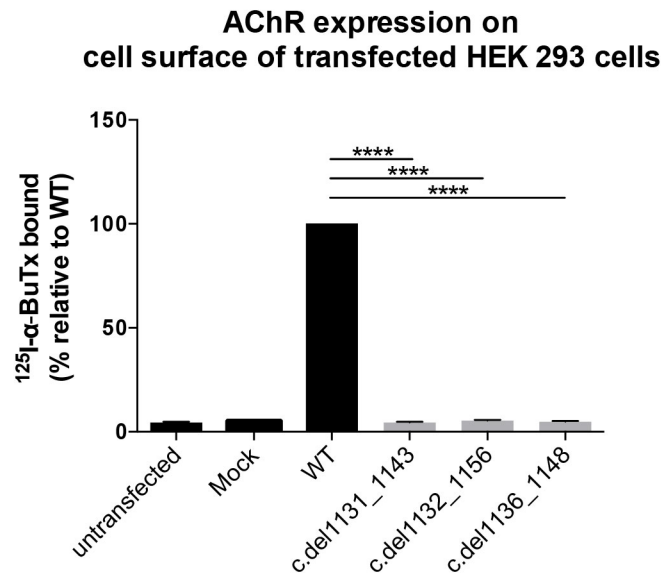


Figure 5-24 ^{125}I - α -BuTx binding to the surface of HEK 293 cells expressing AChR with δ -variants identified from KO6

WT- or mutant- (c.del1131_1143, c.del1132_1156, or c.del1136_1148) δ subunits were transfected with WT- α -, β - and ϵ -subunits into cells at a ratio of 2 α :1 β :1 δ :1 ϵ by PEI transfection method. EGFP was cotransfected with AChR as a transfection efficiency marker and successful transfection was confirmed by fluorescence microscopy. Mock = cells transfected with EGFP only. The results (n=3) are presented as mean percentages $\pm$ SEM relative to cells expressing WT-AChR. One-way ANOVA and Dunnett's multiple comparisons test, asterisks ****p<0.0001.

In comparison to cells overexpressing WT-AChR, the relative ^{125}I - α -BuTx bound on the surfaces of cells expressing δ c.del1131_1143, δ c.del1132_1156 or δ c.del1136_1148 mutant-AChR was significantly reduced to the background levels (Figure 5-24). Therefore the δ -variants are expected to be non-functional and analyses of the cell line KO6 were carried out to confirm its *Chrnd*-KO properties.

5.7.1 Protein expression of δ -subunit in KO6 cells

Cell lysates from the myotubes of untreated C2C12, control 2, control 11 and KO6 were resolved by western blotting with antibodies against δ -subunit and α -tubulin (n=3). The antibody for δ -subunit was raised against a peptide sequence within the 50 amino acid range 490-540 of the human δ -subunit, which is not present on the δ c.del1136_1148, δ c.del1131_1143 and δ c.del1132_1156 variants. Therefore the antibody is expected to detect no δ -subunit from KO6. The ratios of δ -subunit: α -tubulin were analysed, and the results were normalised to control 2.

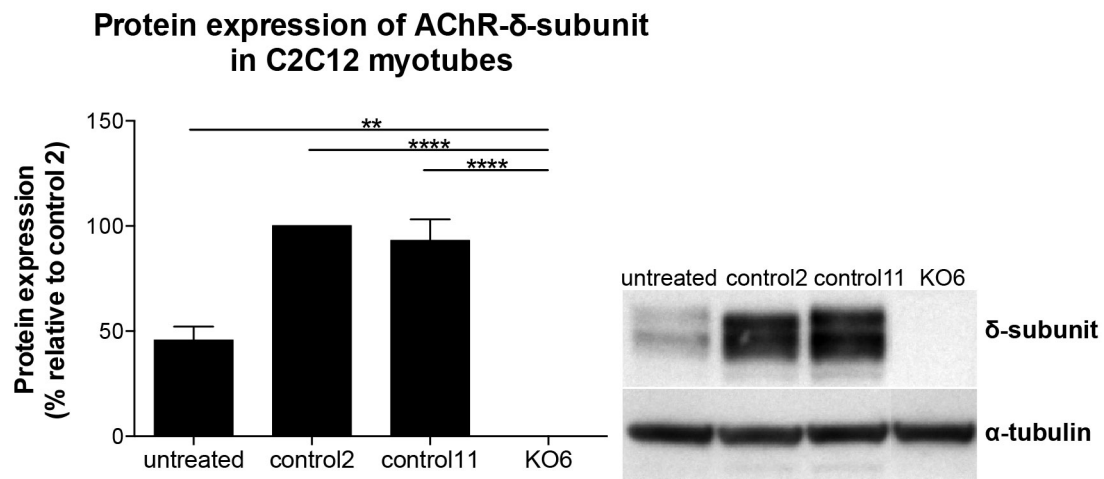


Figure 5-25 Protein expression of AChR δ -subunit in KO6

Cell lysates from untreated C2C12, CRISPR-control 2, 11 and KO6 myotubes was resolved by western blotting analysis using antibodies against δ -subunit and α -tubulin.

The results obtained by densitometry analysis (ratio: δ -subunit: α -tubulin), n=3, are presented as percentages $\pm$ SEM relative to control 2. One-way ANOVA and Tukey's multiple comparisons test, asterisks ****p<0.0001, **p<0.01.

There was no detectable δ -subunit in KO6, confirming that no full length, i.e. WT, δ -subunit, was present and that the antibody can be used in future experiments to specifically detect exogenous δ -subunit overexpressed in these cells (Figure5-25).

5.7.2 Cell surface expression of AChR on KO6 cells

The mutations in the δ -subunit, c.del1136_1148, c.del1131_1143 and c.del1132_1156, prevented cell surface expression of AChR in HEK 293 cells. To confirm that the finding is consistent in C2C12, the relative levels of ^{125}I - α -BuTx binding on myoblasts and myotubes of KO6 were investigated (n=3).

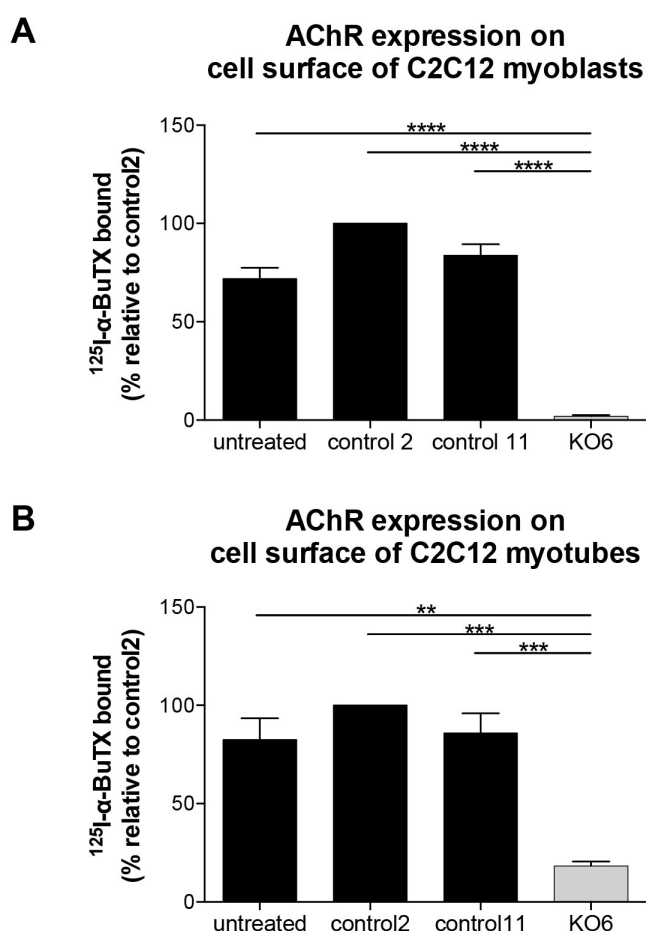


Figure 5-26 ^{125}I - α -BuTx binding to the surfaces of KO6 myoblasts and myotubes

The cell surface expression of AChR on δE381K mutant **(A)** myoblasts and **(B)** myotubes, of KO6 were measured by the ^{125}I - α -BuTx binding assay and compared to untreated C2C12 and CRISPR-control cells, controls 2 and 11.

Results (n=3) were normalised to the levels of ^{125}I - α -BuTx bound on the surfaces of control 2. Each bar represents the mean percentages of ^{125}I - α -BuTx bound to myotube surfaces $\pm$ SEM. One-way ANOVA and Tukey's multiple comparisons tests, asterisks ****p<0.0001, ***P<0.001, **p<0.01.

The relative levels of ^{125}I - α -BuTx bound on the cell surface of KO6 myoblasts was minimal, and was found to be significantly less than myoblasts of untreated C2C12, control 2 and control 11. (Figure 5-26 **A**)

The cell surface expression of AChR on KO6 myotubes also had significantly reduced levels of ^{125}I - α -BuTx bound in comparison to untreated C2C12, control 2 and control 11, but unexpectedly, a low level of ^{125}I - α -BuTx binding was detected on the myotubes of KO6 (Figure 5-26 **B**).

In addition to AChR pentamers, α -BuTx binds with high affinity to the α -subunit of AChR and dimers of α/γ and α/δ subunits (Blount and Merlie, 1989; Tzartos and Changeux, 1983). Hence it was hypothesised that the ^{125}I - α -BuTx bound on KO6 myotubes may have been derived from cell debris, which exposed intracellular AChR subunits. To evaluate this hypothesis, EGFP was overexpressed in KO6 cells, and differentiated myotubes of KO6-expressing EGFP were labelled with the fluorescent α -BuTx-594 conjugate to qualitatively assess α -BuTx-594 binding on these cells.

Intense α -BuTx-594 labelling was visible, but exclusively to cell debris or myotubes that seemed to have been mechanically disrupted (Figure 5-27). Therefore the detected ^{125}I - α -BuTx bound on KO6 were unlikely to represent AChR expression on the cell surface. These results highlight that data from ^{125}I - α -BuTx binding to cultured myotubes should be interpreted with caution.

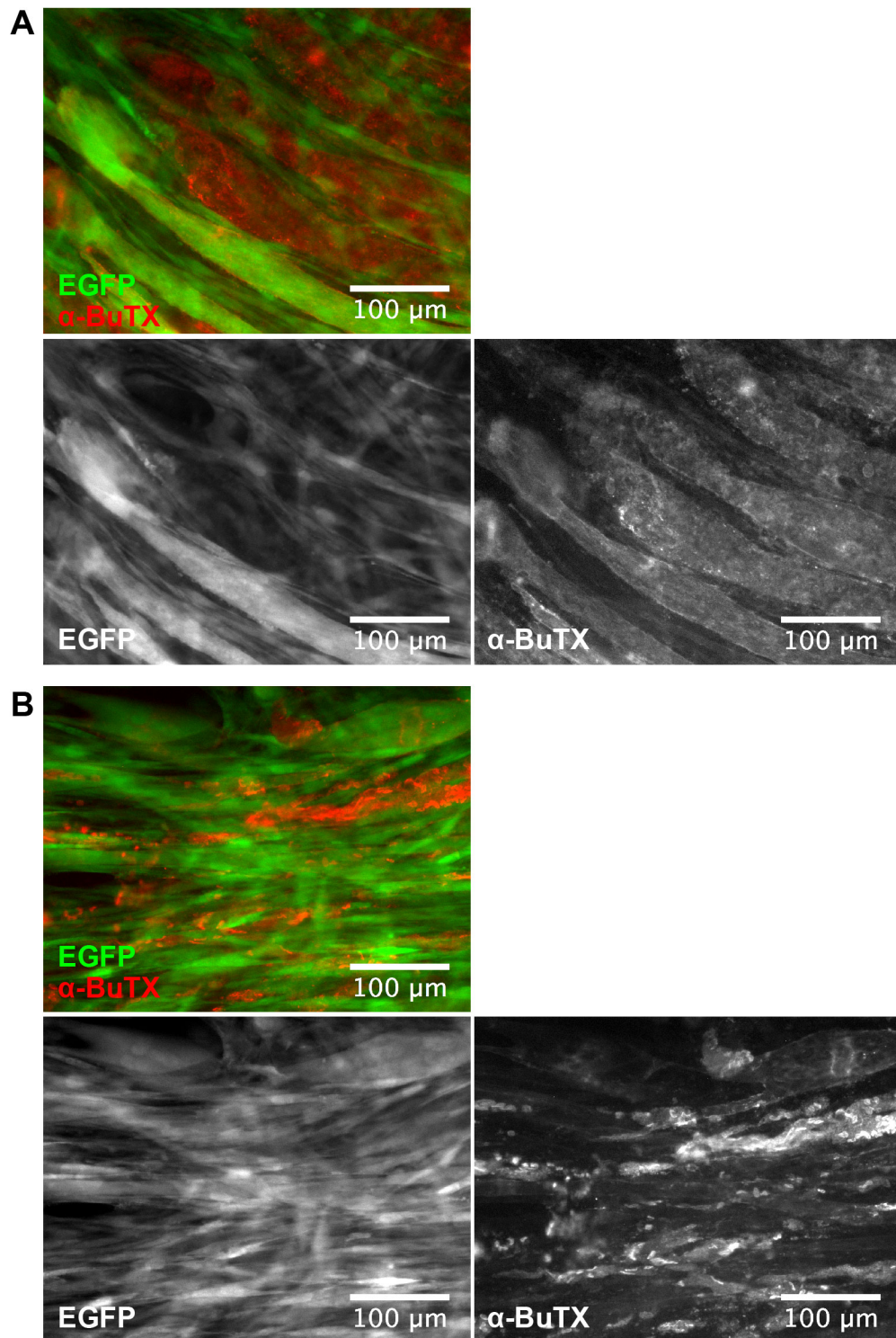


Figure 5-27 Binding of α -BuTx-594 to cell debris of KO6 myotubes

(A) The region of α -BuTx-594 labelling (red) had the morphology of myotubes but no EGFP expression (green) was present, suggesting the mechanical disruption to myotubes exposed intracellular components.

(B) α -BuTx-594 labelling (red) was irregular and intense, which is likely to have derived from the debris of over-differentiated myotubes.

5.7.3 Agrin-induced AChR clustering in KO6 cells

A further control experiment was conducted to confirm that no AChR clusters were formed on myotubes of KO6 after incubation with 1 in 100 FLagrin.

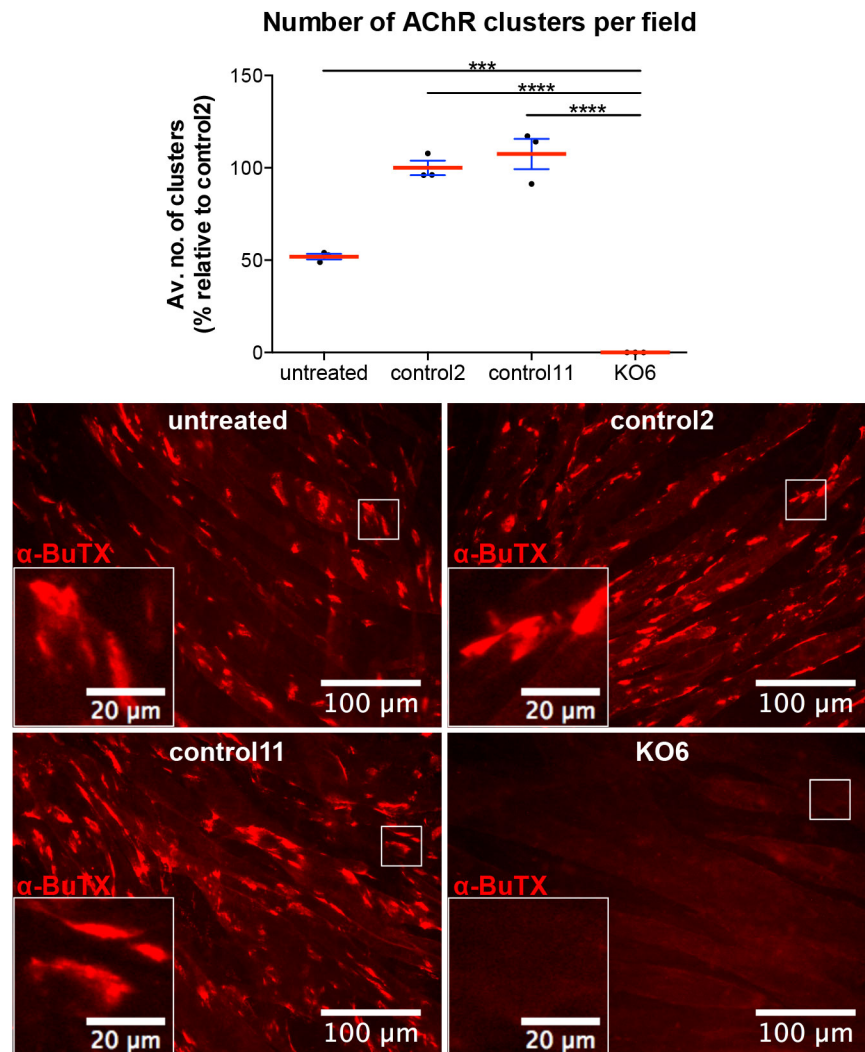


Figure 5-28 Agrin-induced AChR clustering on KO6 myotubes

Myotubes of untreated C2C12, control 2 and control 11 and KO6 were incubated with 1 in 100FLagrin for 16 hours to induce AChR clustering, and subsequently labelled with α -BuTx-594. 60 images from 3 experiments were analysed for each cell line. Each data point represents the mean from 20 images, and the results are normalised to control 2 (mean percentage $\pm$ SEM). One-way ANOVA and Tukey's multiple comparisons test, asterisks **** p <0.0001; *** p <0.001.

Thus KO6 was established as an AChR δ -subunit 'knockout' cell line (Figure 5-28).

5.8 Analysis of δ E381K overexpressed in the *Chrnd*-knockout cell line KO6

cDNA encoding δ WT or δ E381K was introduced to KO6 cells using the pBabe-PURO retroviral system. EGFP that was not tagged to the δ -subunit was also expressed to confirm successful retroviral infection.

5.8.1 Expression of δ -subunits in KO6 cells

The expression levels of δ -subunits in KO6 are dependent on infection efficiency. To identify KO6 cells with comparable expression levels of δ WT or δ E381K, a gradient of concentrations of δ WT retroviruses was used to infect cells: WT1-10%, WT2-20%, WT4-40%, WT6-60%, WT8-80% and WT10-100%; and 100% δ E381K retroviruses were used to introduce the expression of the δ -subunit in these cells (Figure 5-29 A). This was performed so that the any differences observed from the experiments are the effects of the mutation. The levels of δ -subunit in myotubes were analysed by western blotting with antibodies against δ -subunit, GFP and α -tubulin, and the results obtained by densitometric analysis were normalised to WT10.

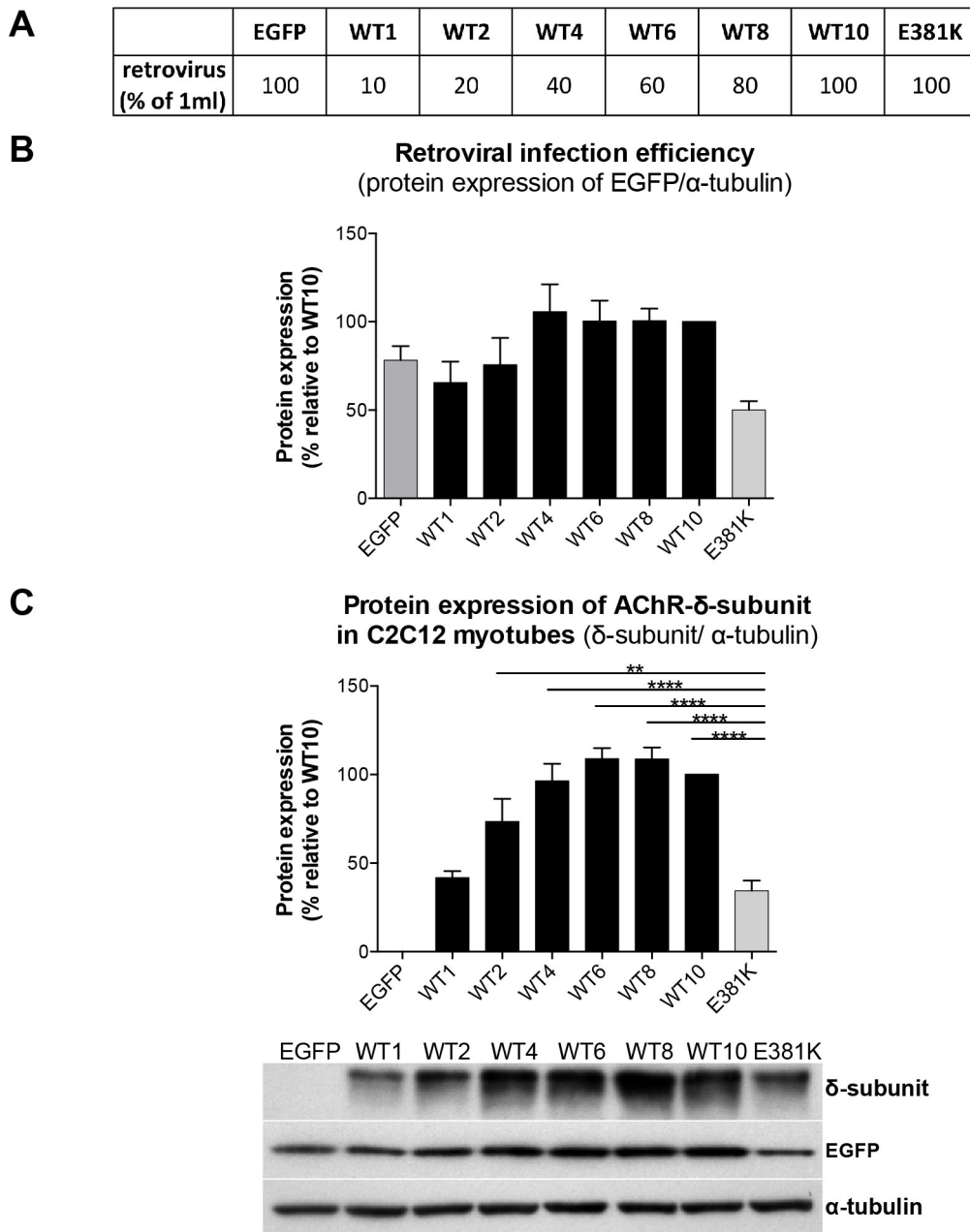


Figure 5-29 Protein expression of δ -subunits when overexpressed in KO6

Whole cell lysates from KO6 myotubes overexpressing δ WT or δ E381K, with untagged EGFP, were resolved by western blotting with antibodies against δ -subunit, GFP and α -tubulin.

(A) A gradient of concentrations of δ WT retroviruses was used to infect KO6

(B) The ratio of EGFP: α -tubulin represents transfection efficiency in myotubes

(C) The ratio δ -subunit: α -tubulin represents the expression levels of δ -subunit

The results obtained by densitometry analysis (n=3) are presented as percentages $\pm$ SEM relative to WT10. One-way ANOVA and Dunnett's multiple comparisons test, asterisks ****p<0.0001, **p<0.01.

To evaluate the relative infection efficiencies, the ratios of EGFP: α -tubulin in myotubes were analysed. Myotubes of KO6 that were infected by higher concentrations of WT retroviruses had slightly higher levels of EGFP than myotubes expressing δ E381K, but the differences were not significant (Figure 5-29 **B**).

The ratio of δ -subunit: α -tubulin suggested significantly higher levels of δ -subunit in the myotubes of WT2, WT4, WT6, WT8 and WT10 when compared to myotubes expressing δ E381K. As WT1 had a similar level of δ -subunit to myotubes overexpressing δ E381K, it was chosen as the control for subsequent experiments (Figure 5-29 **C**).

In summary, the analysis of the protein expression of δ -subunits when overexpressed in KO6 has identified WT1 to be a suitable control for protein expression to evaluate the pathogenic effects of δ E381K.

5.8.2 Agrin-induced AChR clustering on KO6 myotubes expressing δ E381K

AChR clustering on KO6 myotubes was analysed and compared to the established control WT1 that was found to have a similar protein expression to myotubes expressing δ E381K. An additional control WT6 was also included, as it was found to have a similar level of ^{125}I - α -BuTx bound on cell surface of myoblasts in comparison to cells expressing δ E381K (Figure 5-30).

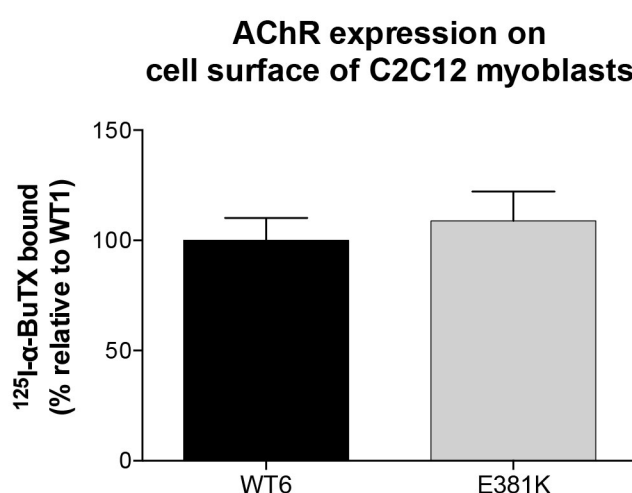


Figure 5-30 WT6 as a control for the cell surface level of AChR of KO6

The levels AChR on cell surface of KO6 myoblasts overexpressing the δ -subunit were measured by the ^{125}I - α -BuTx binding assay. WT6 was found to have similar level of AChR to myoblasts overexpressing δ E381K. Unpaired t test.

Thus WT6 was used as a control for the effects of the levels of cell surface AChR on AChR clustering.

Figure 5-31 shows the representative images of AChR clusters formed on KO6 myotubes expressing δ WT and δ E381K. The quantified data comparing the properties of AChR clusters formed are shown in Figure 5-32.

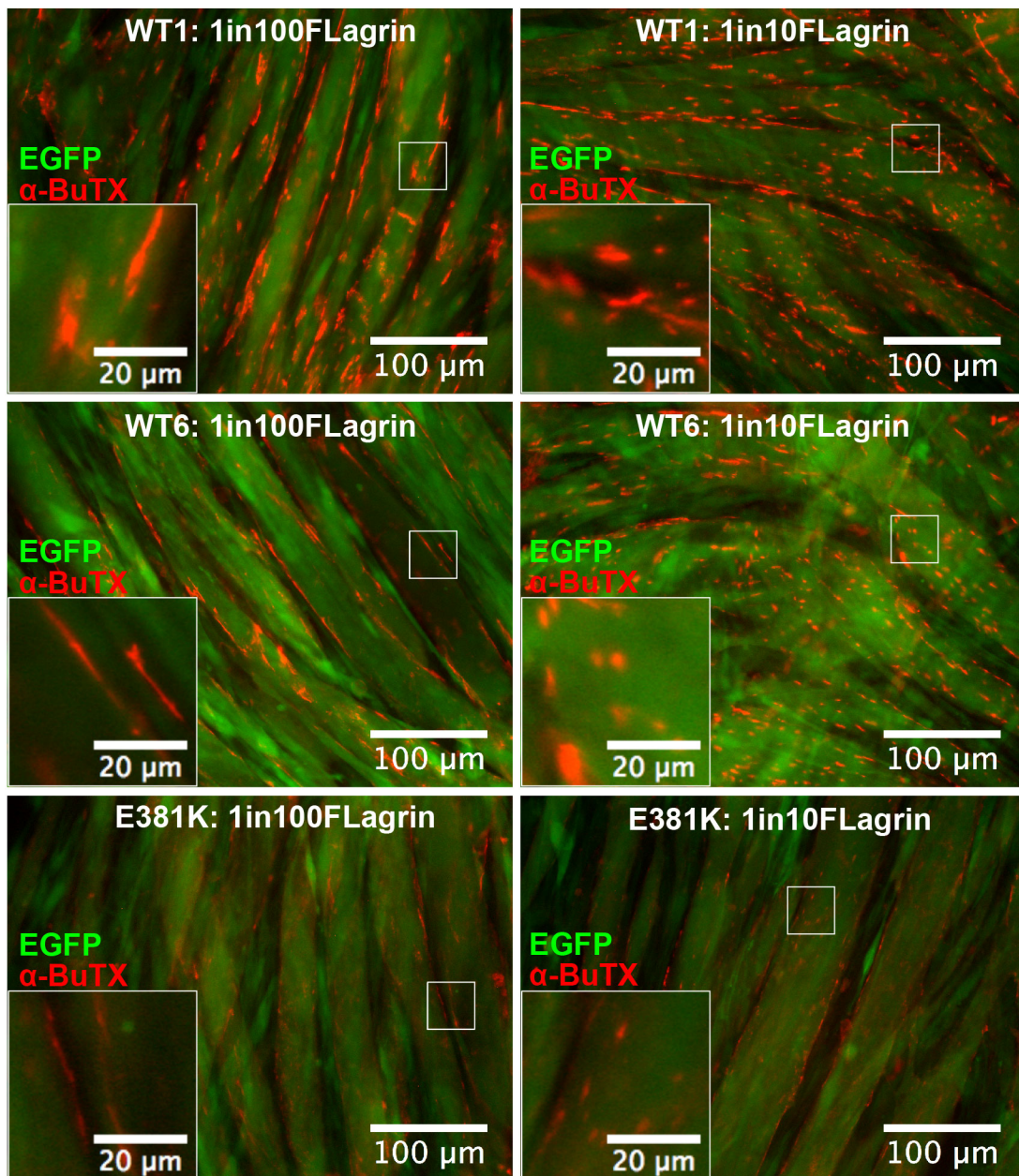


Figure 5-31 Representative images of FLa-grin-induced AChR clustering on KO6 myotubes expressing δ WT or δ E381K

Myotubes of KO6 expressing δ WT or δ E381K were incubated with either 1 in 100FLa-grin or 1 in 10FLa-grin for 16 hours to induce AChR clustering. AChR clusters formed were labelled with α -BuTx-594. WT1 represents the control for the protein expression of δ -subunit, and WT6 represents the control for the levels of cell surface AChR.

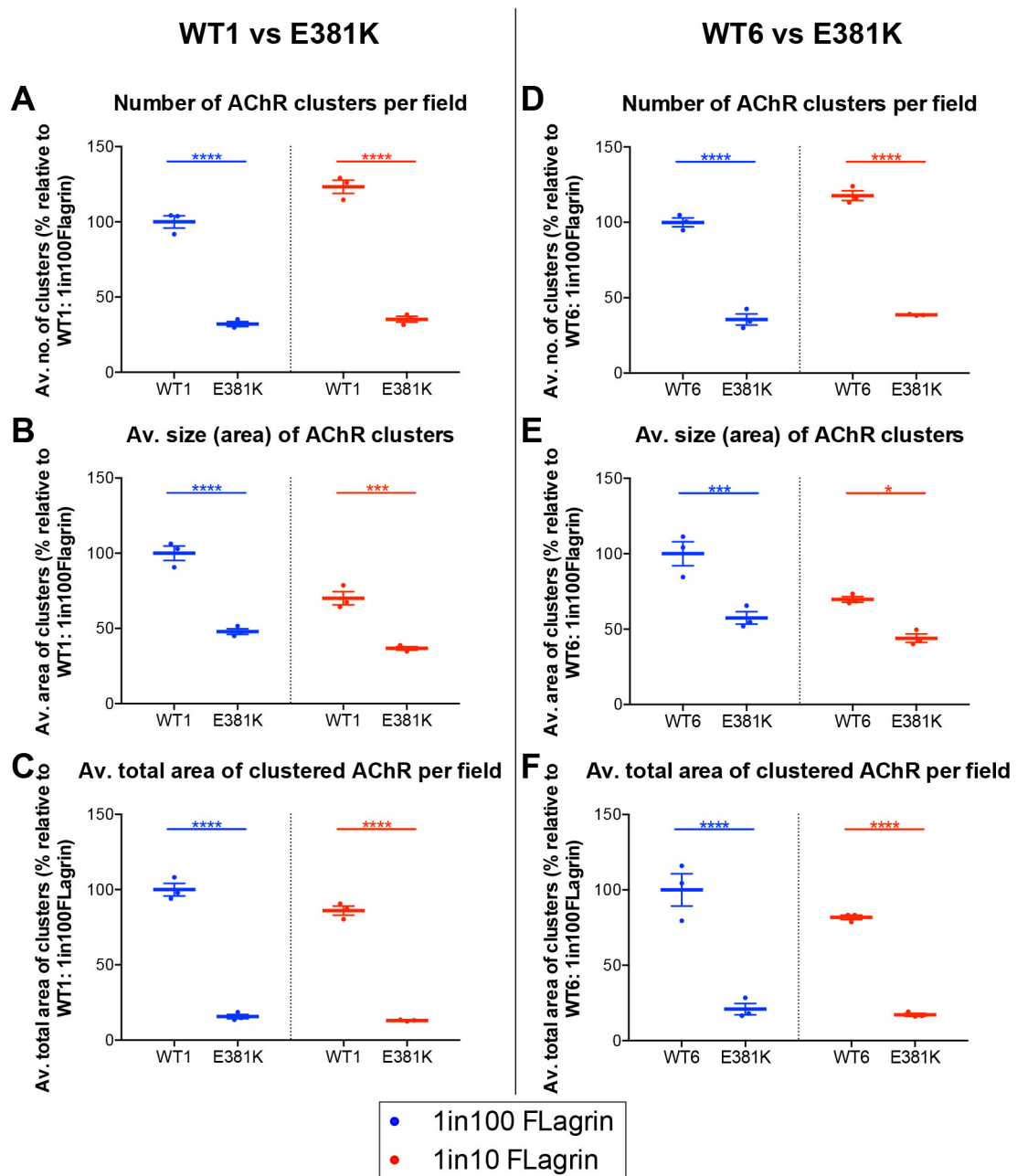


Figure 5-32 Quantified data on agrin-induced AChR clustering on KO6 myotubes overexpressing δ E381K

KO6 overexpressing δ WT or δ E381K were differentiated into myotubes and incubated with either 1 in 100 FLagrin (blue) or 1 in 10 FLagrin (red) for 16 hours to induce AChR clustering. AChR clusters formed were labelled with α -BuTx-594.

60 images from 3 experiments were analysed for each cell line. Each data point represents the mean from 20 images, and the results are presented as mean percentage $\pm$ SEM relative to WT1 (control for protein expression of δ -subunit) or WT6 (control for cell surface AChR expression). Unpaired t tests, asterisks **** p <0.0001; *** p <0.001, * p <0.05.

The number of AChR clusters, induced by 1 in 100 FLagrin and 1 in 10FL, on KO6 myotubes expressing δ E381K were significantly less than WT1 and WT6 (Figure 5-32 **A** and **D**).

The average area of AChR clusters induced by 1 in 100FLagrin and 1 in 10FL on myotubes expressing δ E381K were found to be significantly smaller than WT1 and WT6 (Figure 5-32 **B** and **E**).

The total areas of clustered AChR induced by 1in100FLagrin and 1in10FL on myotubes expressing δ E381K were also significantly smaller when compared to WT1 and WT6 (Figure 5-32 **C** and **F**).

Therefore the results from comparisons made between myotubes expressing δ E381K and the established controls for the protein level of δ -subunits (WT1) and levels of AChR on cell surface (WT6) confirmed, once again, that the mutation E381K reduce the number, size and total area of the AChR clusters formed, and the pathogenic effect is not dependent on the concentration of FLagrin.

5.8.3 Stability of AChR clusters formed on KO6 myotubes expressing δ -subunits of AChR

Thus far the full-length human agrin (FLagrin) has been used to induce AChR clustering for the analysis of AChR clusters formed by mutant-rapsyn-EGFP or mutant δ E381K AChR. The C-terminal part of agrin is sufficient for inducing AChR clustering activities (Gesemann et al., 1995; Hoch et al., 1994), and studies have shown that AChR clusters that were induced by this C-terminal fragment of agrin (C-agrin) were lost upon agrin withdrawal from culture medium (Camilleri et al., 2007; Cossins et al., 2006). In the following experiment, C-agrin was used to investigate the stability of AChR clusters formed on KO6 overexpressing δ WT or δ E381K.

5.8.3.1 AChR clustering induced by rat C-terminal agrin

Myotubes expressing δ WT (WT1 and WT6) or δ E381K were incubated with C-agrin for 16 hours to induce AChR clustering. As a control experiment to evaluate the stability of the effects of C-agrin, the culture medium containing C-agrin was left on myotubes for a further 12 hours. The cells were labelled with α -BuTx-594 and fixed at 0, 1.5, 3, 6 and 12 hours after the initial 16 hours of agrin treatment. For each cell line, a total of 60 microscopic fields from 3 separate experiments were analysed for every time point and the results are presented as percentages relative to time point 0 hours for each respective cell line. Representative images of parts of microscopic fields for AChR clusters that remained at 0, 6 and 12 hours after C-agrin withdrawal can be found in Figure 5-33.

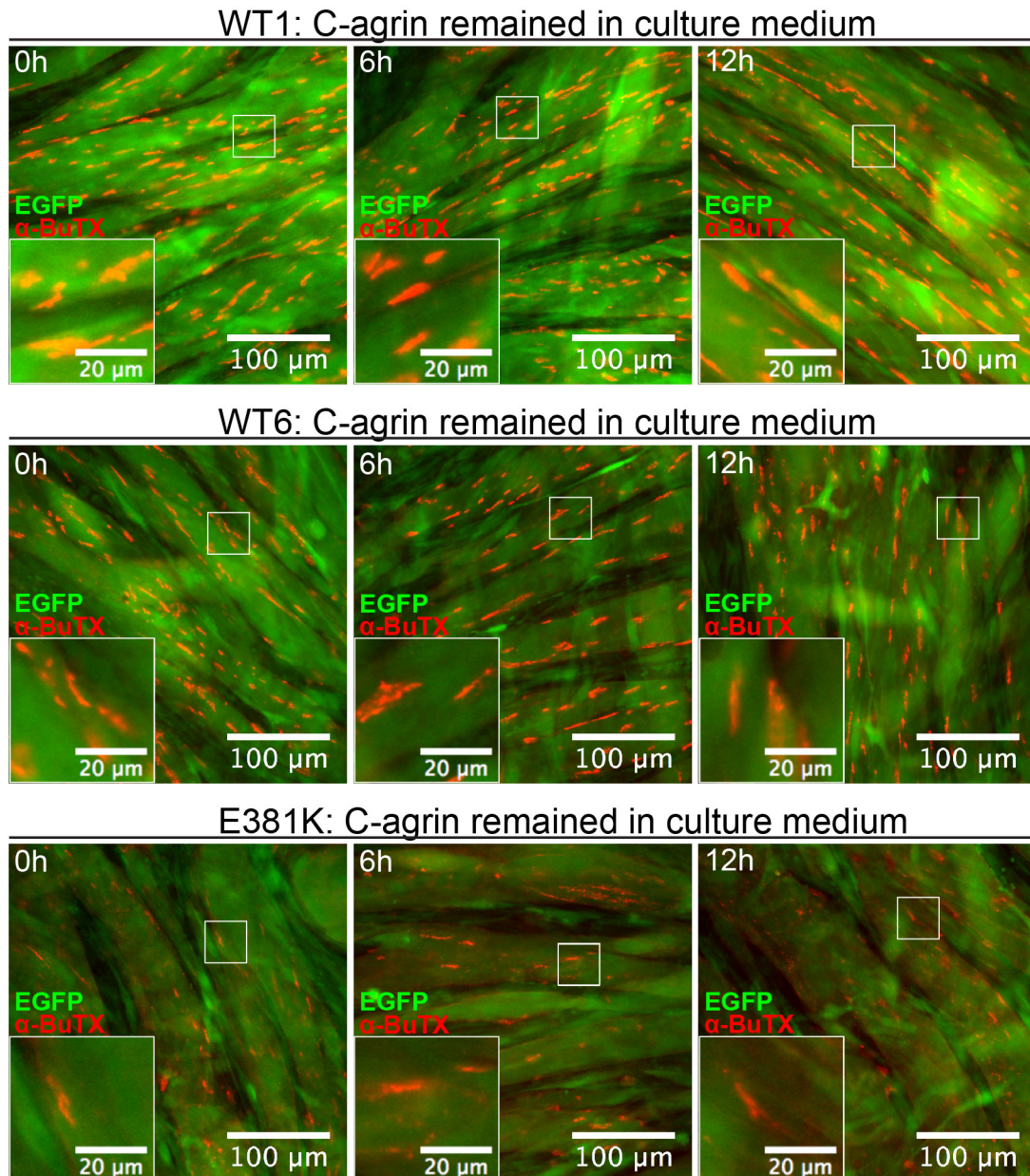


Figure 5-33 Representative images of C-agrin-induced AChR clusters on KO6 myotubes overexpressing δ WT or δ E381K over time

KO6 overexpressing δ WT or δ E381K were differentiated into myotubes and incubated with 1in100C-agrin for 16 hours to induce AChR clustering. Subsequently, C-agrin was left in the culture medium for a further 0, 1.5, 3, 6 and 12 hours, after which AChR clusters were labelled with α -BuTx-594. WT1 represents the control for protein expression of δ -subunit and WT6 represents the control for cell surface AChR expression. Example images represent part of a microscopic field at 0, 6 and 12 hours.

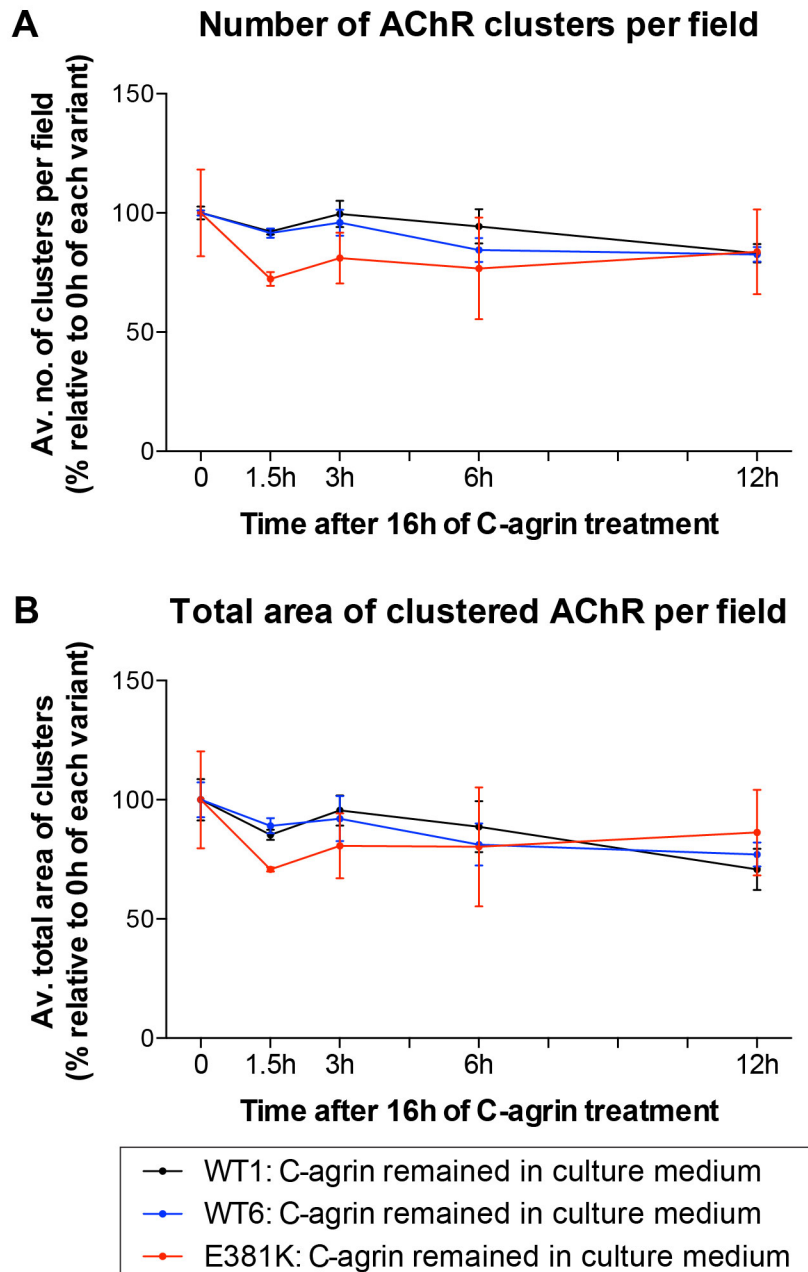


Figure 5-34 Quantified data on C-agrin-induced AChR clusters on KO6 myotubes overexpressing δ WT or δ E381K over time

KO6 overexpressing δ WT or δ E381K were differentiated into myotubes and incubated with 1 in 100 C-agrin for 16 hours to induce AChR clustering. Subsequently, C-agrin was left in the culture medium for a further 0, 1.5, 3, 6, and 12 hours, after which AChR clusters were labelled with α -BuTx-594.

60 images from 3 experiments were analysed and the data obtained were normalised to 0 hours for each condition as mean percentages $\pm$ SEM. WT1 represents the control for protein expression of δ -subunit and WT6 represents the control for cell surface AChR expression. Two-way ANOVA with Tukey's multiple comparisons tests

After the initial 16 hours of C-agrin stimulation, a further 12 hours of exposure to C-agrin did not affect the relative number and total area of clustered AChR that remained on myotubes expressing δ WT and δ E381K when compared to their respective maximum values at 0 hours (Figure 5-34).

At each time point, the relative number and total area of AChR clusters, relative to their respective original maximum amounts, that remained on the myotubes overexpressing δ WT and δ E381K were similar (Figure 5-34).

Thus the results suggest that after 16 hours of C-agrin to stimulate AChR clustering, the AChR clusters formed are stable for a further 12 hours in the presence of C-agrin.

5.8.3.2 Stability of AChR clusters upon the removal of C-agrin

The stability of AChR clusters was evaluated by the withdrawal of C-agrin, by 3 changes of culture medium, from KO6 myotubes overexpressing δ WT or δ E381K after the 16 hours of incubation with C-agrin to induce AChR clustering. Again, the myotubes were labelled with α -BuTx-594 at 0, 1.5, 3, 6 and 12 hours after C-agrin withdrawal. For each of the time points, 60 images from 3 experiments were analysed, and the results were normalised to the maximum clustering at 0 hours for each of the respective cell lines. Representative images of the AChR clusters present on myotubes at 0, 6, and 12 hours are shown in Figure 5-35.

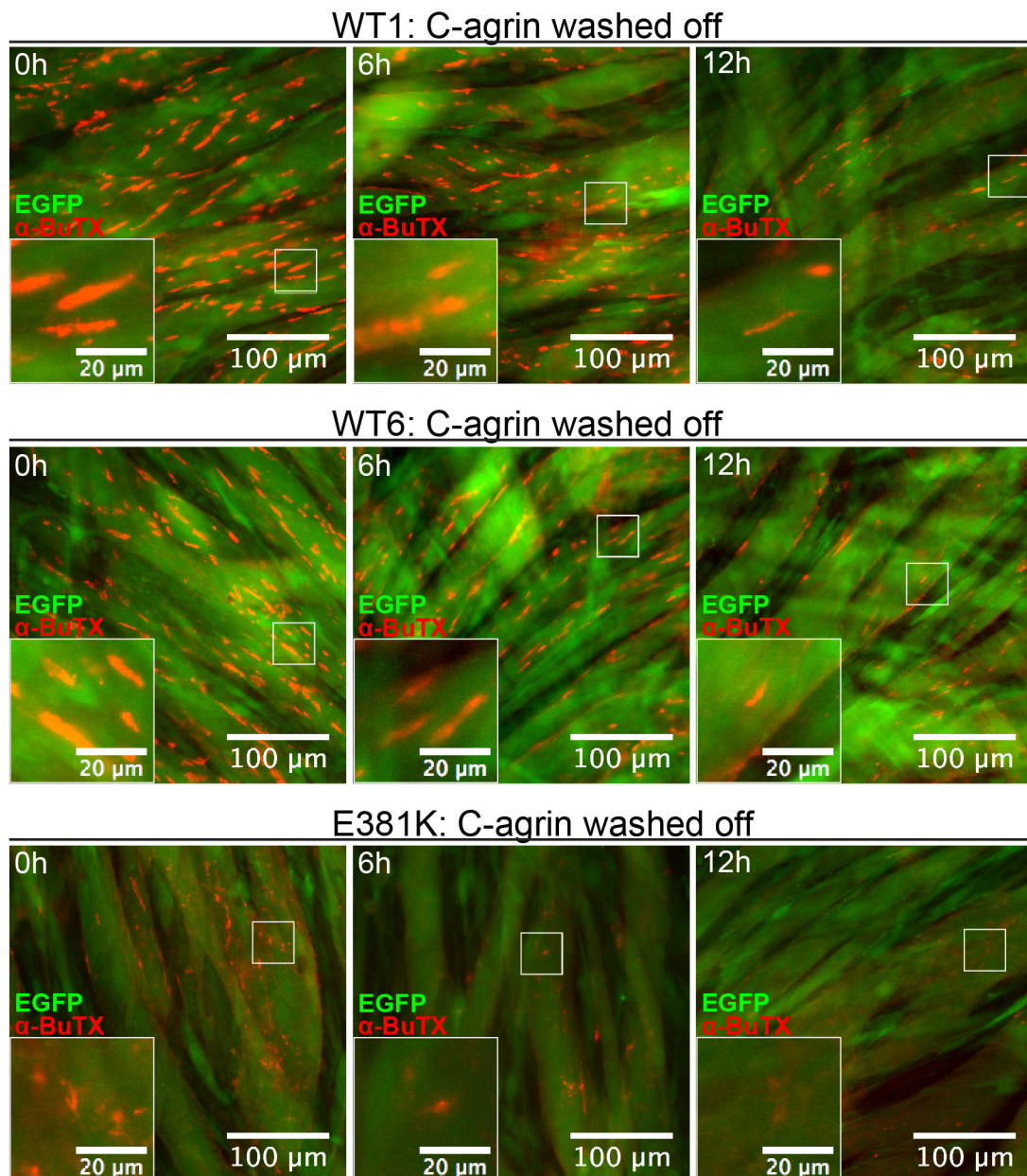


Figure 5-35 Representative images of AChR cluster loss on KO6 myotubes overexpressing δ WT or δ E381K after C-agrin withdrawal

KO6 overexpressing δ WT or δ E381K were differentiated into myotubes and incubated with 1 in 100 C-agrin for 16 hours to induce AChR clustering. Subsequently, C-agrin was withdrawn from the cells by changing the culture medium 3 times, and AChR clusters were labelled with α -BuTx-594 at 0, 1.5, 3, 6 and 12 hours agrin removal. WT1 represents the control for protein expression of δ -subunit and WT6 represents the control for cell surface AChR expression. Example images represent part of a microscopic field at 0, 6 and 12 hours.

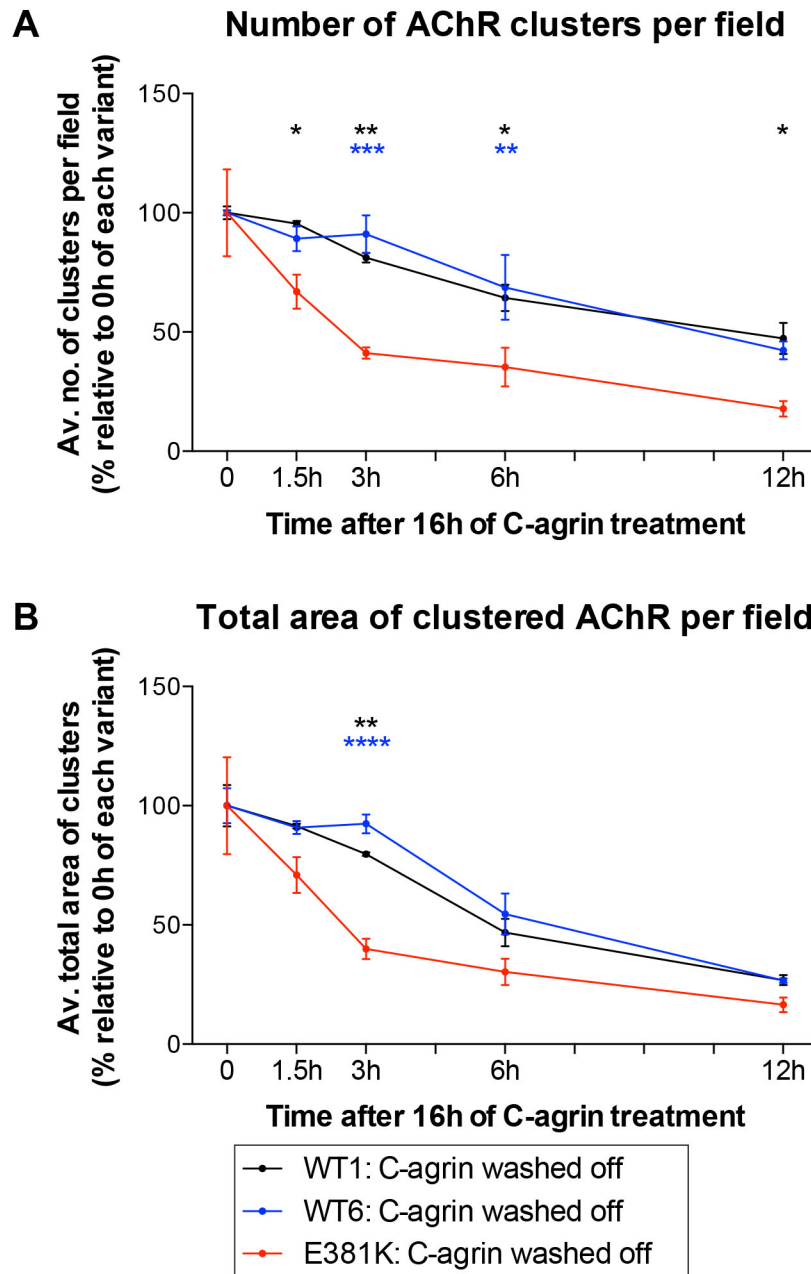


Figure 5-36 Quantified data on AChR cluster loss on KO6 myotubes overexpressing δ WT or δ E381K after C-agrin withdrawal

KO6 overexpressing δ WT or δ E381K were differentiated into myotubes and incubated with 1 in 100 C-agrin for 16 hours to induce AChR clustering. Subsequently, C-agrin was withdrawn from the cells and AChR clusters were labeled with α -BuTx-594 at 0, 1.5, 3, 6 and 12 hours after agrin removal. WT1 represents the control for protein expression of δ -subunit and WT6 represents the control for the levels of cell AChR on cell surface.

60 images from 3 experiments were analysed and the data obtained were normalised to 0 hours for each condition as mean percentages $\pm$ SEM. Two-way ANOVA with Tukey's multiple comparisons tests

After C-agrin withdrawal, the number and total area of AChR clusters that remained on δ WT were stable for the initial 3 hours when compared to their respective maximum values at 0 hours, and became significantly reduced at 6 and 12 hours. By contrast, the number and total area of AChR clusters on myotubes expressing δ E381K became significantly reduced from 1.5 hours. Thus, the results confirmed that the removal of C-agrin caused AChR clusters to disassemble (Figure 5-36).

The relative number of AChR clusters, with respect to the original levels at 0 hours for each cell line, that remained on myotubes expressing δ E381K was significantly lower than WT1 at 1.5 hours after C-agrin was removed, and the significant differences were maintained for the duration of the entire experiment. The differences between WT6 and myotubes expressing δ E381K became significant after 3 and 6 hours of C-agrin removal (Figure 5-36).

The relative total area of clustered AChR that remained on myotubes overexpressing δ E381K was significantly less than WT1 and WT6 at 3 hours after the removal of C-agrin, but the differences were not significant at later time points. (Figure 5-36)

In summary, the results suggest that AChR clusters formed on myotubes expressing δ E381K were lost more rapidly than δ WT, and are therefore unstable.

5.9 Discussion

5.9.1 CRISPR-Cas9n mutagenesis in C2C12

The aim of this chapter was to evaluate the pathogenic properties of the *Chrnd* E381K mutation to provide insights into the underlying mechanisms of AChR deficiency that can be modelled by cell culture studies of AChR clustering. First, model cell culture systems needed to be validated. C2C12 cells that express endogenous δ E381K (A11 and B15) were generated by CRISPR-Cas9n mutagenesis for investigations, but phenotypic variations between control cells hindered data interpretations. Subsequently, a cell line that lacks functional δ -subunits, KO6, was generated, and δ -subunits were overexpressed in these cells to evaluate the defects in AChR clustering caused by the mutation.

5.9.1.1 Targeted missense mutation in *Chrnd*

To date, the use of CRISPR-Cas9n to precisely edit the genome of C2C12 cells has not been demonstrated in literature. The success in the generation of clonal cell lines A11 and B15 has proven the feasibility of using the CRISPR-Cas9n system for investigating specific effects of the *Chrnd* mutation E381K. However, there are several aspects of the design of the system that could be optimised in the future.

Different amounts of CRISPR plasmid (A: 10 μ g or B: 20 μ g), together with a single-stranded oligonucleotide repair template, were electroporated into C2C12 myoblasts for the mutagenesis. PCR analysis and DNA sequencing results have identified the presence of insertion/deletion indels in A $\approx$ 50% and B $\approx$ 45% of the isolated clonal

cell lines. This confirmed that either amounts of CRISPR plasmid were sufficient for Cas9n activities to generate double-stranded DNA breaks at the target site. The Cas9n system possesses high target specificity (Ran et al., 2013a), but high concentrations of Cas9/sgRNA can lead to off-target cleavage of DNA with mismatches close to or in the PAM regions (Pattanayak et al., 2013).

The success rates in generating clones that harbour the mutation were low, $\delta E381K$: A $\approx$ 1.5% and B $\approx$ 2%. Only 1 clone, A11, was homozygous for $\delta E381K$. Other studies have also reported that the frequencies of precise gene editing by homology-directed repair are inherently low in both *in vitro* and *in vivo* systems (Mali et al., 2013b; Yang et al., 2013). The high percentage of cells with unpredicted indels, heterozygous PAM sites in A11 and the truncating mutation in the second allele of B15 suggest that homology directed mutagenesis by the repair template was outcompeted by the non-homologous end joining pathway for DNA repair. Recent research has identified possible strategies to suppress the non-homologous end joining, which could be adapted to the current protocol to increase the efficiency of homology-directed genome editing (Chu et al., 2015; Maruyama et al., 2015). For example, the inhibition of DNA ligase IV, an important enzyme for NHEJ, by Scr7 increased the frequencies of HDR by up to 19-fold (Chu et al., 2015).

The heterozygously mutated PAM sites in the A11 clone that is homozygous for E381K suggests that the screening method by the restriction digestion at the PAM2 site might have missed clones that harboured the mutation E381K. To enhance screening sensitivity, the design of the repair template could be improved by adapting

the GAG>AAA change for E381K instead of the used GAG>AAG (Muller et al., 2006b) to introduce a DraI restriction site at the mutation (Figure 5-37).

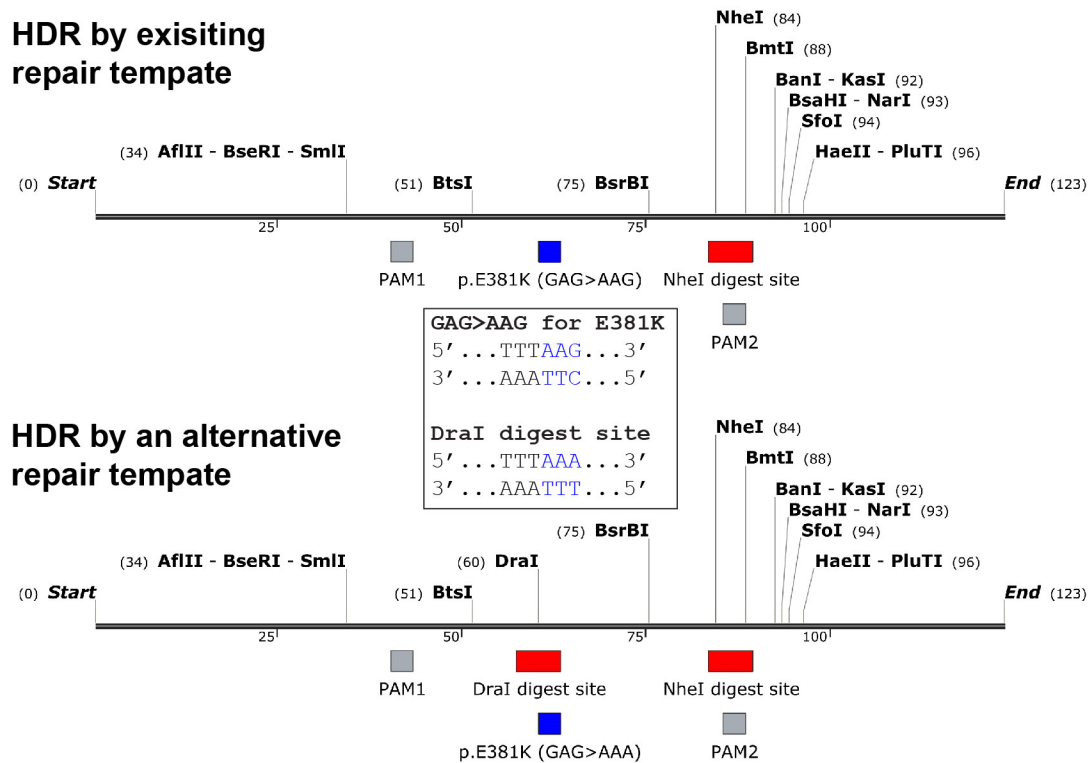


Figure 5-37 Optimised design of repair template to improve screening for CRISPR-Cas9n edited clonal cell lines

Homology directed repair (HDR) by the current single-stranded oligonucleotide gives rise to a restriction site at PAM2, NheI, which is not present in WT DNA. The NheI site detects clones that have mutated PAM2, which is not specific for the mutation E381K.

A repair template that is slightly different to the one used in this thesis, GAG>AAA to introduce the mutation E381K would give rise to a new restriction digest site, DraI, that allows detection for the mutation p.E381K in cells that have gone through homology-directed mutagenesis. Hence this would improve the sensitivity and specificity in detecting mutant clones.

The variable properties between C2C12 clonal cells hindered data interpretation on the functional effects of δ E381K. The control cell lines were chosen based on their similarities to untreated C2C12 cells, but the AChR clustering properties of these cells were different. Moreover, there were differences in the protein expression of δ WT in untreated C2C12 and the selected controls.

There are several possible contributing factors to the variations in the clonal properties of the C2C12 clones. Firstly, the variable abilities for the cells to differentiate into myotubes, as the transcription of AChR subunit genes and the subsequent expression of assembled AChR increase during the process of differentiation (Sanes and Lichtman, 2001). Secondly, C2C12 could be a non-clonal population of cells. A study has identified a population of C2C12 cells, known as 'reserve cells', that do not commit to the differentiation pathway, and the expansion of isolated reserve cells gave rise to a mixed population of reserve cells and cells that would differentiate into myotubes (Yoshida et al., 1998). Furthermore, the likelihood for C2C12 to be a polyploid cell line can also contribute to the variable properties between clonal cells as it has been identified that different ploidies can cause variable patterns of gene expression (Galitski et al., 1999; Hieter and Griffiths, 1999). Thus if C2C12 cells are a polyploid cell line, they may not precisely mimic the mechanisms of the E381K mutation *in vivo*, and the overexpression of δ E381K in a *Chrnd*-KO cell line may be a better approach to gain clearer insights.

5.9.1.2 Functional knockout in *Chrnd*

The relative success rate (~20%) in generating a KO cell line that does not form AChR clusters was much higher in comparison. To date, one study has demonstrated the success of inducing gene KO, of the prion protein, in C2C12 cells by the CRISPR-Cas9 system with a success rate of ~2% (Mehrabian et al., 2014). The higher success rate in my results may be due to the selection of transfected cells by G418 prior to clonal isolation, as the reported study used a CRISPR vector that lacks any selection marker. The 3 variants of truncated δ -subunits in KO6 possess deletions that remove over 2 exons of the genomic DNA, and their expressions may be prevented by nonsense-mediated mRNA decay, a translation-coupled mechanism that prevents the synthesis of truncated proteins (Brognia and Wen, 2009).

5.9.1.3 Off-target activities of Cas9n

Concerns regarding target specificity mostly relate to WT-Cas9 (Cho et al., 2014; Fu et al., 2013; Hsu et al., 2013). The CRISPR/Cas9n offers much higher specificity since the double pair of gRNA are unlikely to bind to the same off-target site to induce double-stranded DNA breaks (Cho et al., 2014; Mali et al., 2013a; Ran et al., 2013a). The CRISPR Design Tool (<http://crispr.mit.edu>) predicted no off-target activities for the pair of guides A and B used. However, a single-base mismatch in the interface between DNA and gRNA, on one of the paired gRNA, can be tolerated by the CRISPR/Cas9n system to induce DSB (Lin et al., 2014). To further enhance specificity, truncated guide sequences of 17 nucleotides in length could be employed in the future (Fu et al., 2014). The direct delivery of purified recombinant Cas9 protein complexed with gRNA into cells was also reported to reduce off-target effects due to the rapid degradation of Cas9 by endogenous proteases (Kim et al., 2014).

5.9.2 Molecular effects of the *Chrnd* mutation E381K

Results from the 3 experimental model systems: the overexpression of δ -subunits in WT-C2C12, the clonal cell lines of C2C12 mutated by CRISPR-Cas9n and the overexpression of δ -subunits in KO6, confirmed that δ E381K reduce agrin-induced AChR clustering. The findings on the molecular consequences of δ E381K are summarised in Table 5-2.

Table 5-2 Summary on the pathogenic effects of δ E381K

	¹ Overexpression of δ E381K in WT-C2C12 cells	² Endogenous expression of δ E381K in C2C12 cells A11 B15		³ Overexpression of δ E381K in KO6 cells	⁴ Overexpression of δ E381K AChR in HEK 293 cells
Steady-state protein expression of δ -subunit of AChR	n/a	Reduced*	Reduced*	n/a	=
Cell surface expression of AChR on myoblasts	n/a	Reduced	Reduced	n/a	Reduced#
AChR clustering (Number of clusters)	Reduced	Reduced	Reduced	Reduced	n/a
AChR clustering (Average size of clusters)	Reduced	Reduced	Reduced	Reduced	n/a
AChR clustering (Total area of AChR clusters)	Reduced	Reduced	Reduced	Reduced	n/a
Stability of AChR clusters	n/a	n/a	n/a	Reduced	n/a

The pathogenic effects (red) were identified based upon comparisons with: ¹WT-C2C12 overexpressing WT- δ , ²Control 2 and control 11 cells, ³KO6 cells overexpressing WT- δ AChR subunits and ⁴HEK 293 transfected to overexpress δ WT-AChR

A11 = C2C12 homozygous for E381K; B15 = C2C12 heterozygous for E381K with a deletion mutation that ablates function

KO6 = C2C12 cells that do not express functional δ -subunit, i.e. a *Chrnd* functional knockout cell line

n/a = not investigated; reduced* = when compared to untreated C2C12 instead of the clonal control cells, and experiments on transfected HEK 293 cells suggested no differences; # = findings from (Muller et al., 2006b)

The *Chrnd* mutation E381K does not affect the protein levels of δ -subunit, but δ E381K was found to reduce the cell surface expression of AChR and AChR clustering. The AChR clusters formed with mutant AChR assembled with δ E381K were less stable in comparison to WT-AChR, which supports the hypothesis that δ -subunit to have roles implicated in the stabilisation of AChR clusters.

5.9.2.1 The E381K mutation in *Chrnd* does not affect protein expression

The overexpression of AChR in HEK 293 cells has shown that the mutation E381K does not affect protein expression. The heterogeneity in the expression levels of δ -subunits in control cell lines and untreated C2C12 myotubes hindered interpretations for the results from mutant cell lines A11 and B15. It has been identified that the nuclei of both myoblast and myotubes generate variable levels of AChR subunit mRNA levels, and the differences exist prior to fusion (Bursztajn et al., 1989; Su et al., 1995). Moreover, the fusion of myoblasts increases the level of AChR subunit gene expressions (Su et al., 1995). Since the differentiation processes can be variable between different C2C12 cells, the levels of AChR are likely to vary according to the condition of the cells and their ability to differentiate into myotubes.

5.9.2.2 The mutant δ E381K reduces the levels of cell surface AChR

The endogenous expression of δ E381K in A11 and B15 reduced the levels of AChR on myoblast cell surfaces to around 50% of the untreated C2C12 cells. The results are in agreement with previous findings for E381K to reduce the cell surface expression of AChR to 70% of controls (Muller et al., 2006b). The lower levels of surface AChR detected from A11/B15 cells may be due to cell heterogeneity in the system.

The detection of AChR cell surface expression on KO6 myotubes was unexpected. It has been reported that in the absence of δ -subunit, $\alpha_2\beta\epsilon_2$ pentamers could be formed intracellularly (Quiram et al., 1999), but minimal ^{125}I - α -BuTx binding was detected on cell surface when these pentamers were expressed in heterologous cells (Quiram et al., 1999; Yu and Hall, 1994a). Hence the low levels of ^{125}I - α -BuTx detected are

likely to be bound to internal partially formed AChR that were exposed from leaky cells upon differentiation into myotubes. Thus the data from ^{125}I - α -BuTx binding to myotubes should be treated with caution. It is also notable that the detected levels of ^{125}I - α -BuTx bound on control myotubes were at least 5-folds higher than KO6 myotubes. Therefore the non-specific binding should not devalue the data on the effects of *RAPSN* mutations towards the levels of surface AChR shown in Chapter 4.

5.9.2.3 The mutant δE381K reduces AChR clustering

Agrin-induced AChR clustering on A11/B15 mutant cells or the overexpression of δE381K in KO6 cells have both identified a clear reduction in the total area of clustered AChR to ~20% of controls. Although the effect was also evident due to the overexpression of δE381K in WT-C2C12, the relative reduction in the total area of clustered AChR was smaller, ~50% compared to myotubes overexpressing δWT , which suggests endogenous δWT was able to partially compensate for the reduced AChR clustering due to E381K. Thus in future, the use of the *Chrnd*-KO cell line may be a better system for determining the pathogenicity of other *CHRND* mutations where their underlying mechanisms are yet to be identified.

The reduced surface expression of AChR could be a contributing factor towards reduced AChR clustering, or it may be that reduced clustering efficiency leads to an increased turnover of surface AChR as synaptic AChR are metabolically more stable than extra-synaptic receptors (Akaaboune et al., 2002).

5.9.2.4 The mutant δ E381K reduces the stability of AChR clusters

C-agrin was used to induce AChR clustering and was subsequently removed to analyse the stability of the clusters formed. The choice of C-agrin in this experiment was based on the properties of this fragment in comparison to FLagrin. As shown in Chapter 4, the effect of FLagrin is stable for at least 40 hours, whereas C-agrin is likely to be less stable. In *Rapsn*-KO myotubes overexpressing rapsyn-EGFP, C-agrin was unable to induce AChR clustering, and therefore FLagrin was used in those experiments. It would be of interest in the future to also evaluate the stability of AChR clusters, in the presence of FLagrin, by carbachol-induced AChR disassembly.

Upon C-agrin withdrawal, the δ E381K-AChR clusters on KO6 myotubes disassembled much more rapidly when compared to the WT-AChR controls, demonstrating that the mutation on an AChR subunit affects the stability of AChR clusters. This could be due to an effect on the stability of the AChR δ -subunit, or impaired interactions between the receptors and rapsyn. To determine if the latter regulates the stability of AChR clusters, a *Chrnd/Rapsn*-double knockout cell line could be generated by the CRISPR-Cas9n system and the interactions between exogenous δ E381K-AChR and rapsyn-EGFP could be investigated by microscopy.

Potentially the E381K mutation may also affect PKC phosphorylation of the δ -subunit, which was previously found to associate with dispersal of AChR clusters (Nimnual et al., 1998). It has been reported that stimulation of PKC accelerates the turnover of AChR from clusters *in vivo* (Martinez-Pena y Valenzuela et al., 2013).

Chapter 6

A pilot study on the potential use of salbutamol for AChR deficiency syndrome

6.1 Introduction

Current treatments for CMS patients with AChR deficiency syndromes due to *RAPSN* or AChR subunit mutations rely on cholinergic agonists to enhance neuromuscular transmission, and therefore to improve muscle strength. Pyridostigmine is most commonly used as the first line of treatment (Engel et al., 2015; Finlayson et al., 2013). 3,4-DAP can be used alone or combined with pyridostigmine for optimal effects (Engel et al., 2015; Finlayson et al., 2013; Maselli et al., 2003; Ohno et al., 2002). However, these treatments have limitations. Although fast-acting, the beneficial effects of such treatments are short-lived and often require long-term administration for the duration of the disease course (Finlayson et al., 2013). It has been reported that the beneficial effects of cholinesterase inhibitors deteriorate in long-term treatment regimens (Rodríguez Cruz et al., 2015), leaving patients with continued muscle weakness. Furthermore, most patients only partially and some do not respond to such therapies (Milone et al., 2009; Ohno et al., 2002; Sadeh et al., 2011). Hence additional treatment strategies that address such unresolved issues would be desirable to improve the prognosis for AChR deficiency patients.

Recently, the use of β_2 -adrenergic receptor agonists such as ephedrine or salbutamol, in combination with pyridostigmine and 3,4-DAP, has been reported to provide additional benefits for 8 CMS patients with AChR deficiency due to mutations in

CHRNE (Rodríguez Cruz et al., 2015; Sadeh et al., 2011). The β_2 -adrenergic receptor agonists are effective and used as primary treatments for CMS caused by mutations in *DOK7* and *COLQ* (Engel et al., 2015; Finlayson et al., 2013; Lashley et al., 2010; Witting and Vissing, 2014). However, it should be noted that treatments that benefit one type of CMS could be ineffective or have detrimental effects in another type (Engel et al., 2015). For example, anticholinesterase treatments have been reported to worsen conditions for *DOK7*-CMS patients (Witting and Vissing, 2014).

The mechanisms through which ephedrine or salbutamol act to benefit CMS patients are not known. The β_2 -adrenergic receptor belongs to the family of G-protein-coupled receptors, and is well expressed in skeletal muscles (Kim et al., 1991). The effects of β_2 -adrenergic receptor agonists are generally believed to be mediated by the activation of the G-protein Gs, which in turn activates adenylate cyclase to increase the intracellular levels of cyclic adenosine monophosphate (cAMP) (Benovic, 2002). Of note, activity of the cAMP-dependent protein kinase A (PKA) has been shown to modulate the clustering and stability of AChR clusters *in vivo* (Choi et al., 2012a; Martinez-Pena y Valenzuela et al., 2013; Perkins et al., 2001; Roder et al., 2010; Roder et al., 2012). Hence ephedrine or salbutamol may act to stabilise postsynaptic AChR clusters via cAMP-PKA signaling or other kinase pathways.

A mouse model of AChR deficiency syndrome ($h\gamma^+\epsilon^{-/-}$) is available in our laboratory for use (Cossins et al., 2004). It has been demonstrated that *Chrne*-KO, $\epsilon^{-/-}$, mice die between 8 to 14 weeks of age due to the lack of adult AChR $\alpha_2\beta\delta\epsilon$ (Missias et al., 1997). The introduction of human AChR γ -subunit ($h\gamma$) expression under regulations by the human skeletal α -actin promoter in mice with the $\epsilon^{-/-}$ background allows $h\gamma^+\epsilon^{-/-}$

mice to live into adulthood (Cossins et al., 2004). Figure 6-1 illustrates the AChR pentamers expressed in $h\gamma^+\epsilon^{-/-}$ mice. They display features that are similar to AChR deficiency syndrome in humans, with fatigable muscle weakness, reduced number of AChR at the NMJ and defective neuromuscular transmission (Cossins et al., 2004).

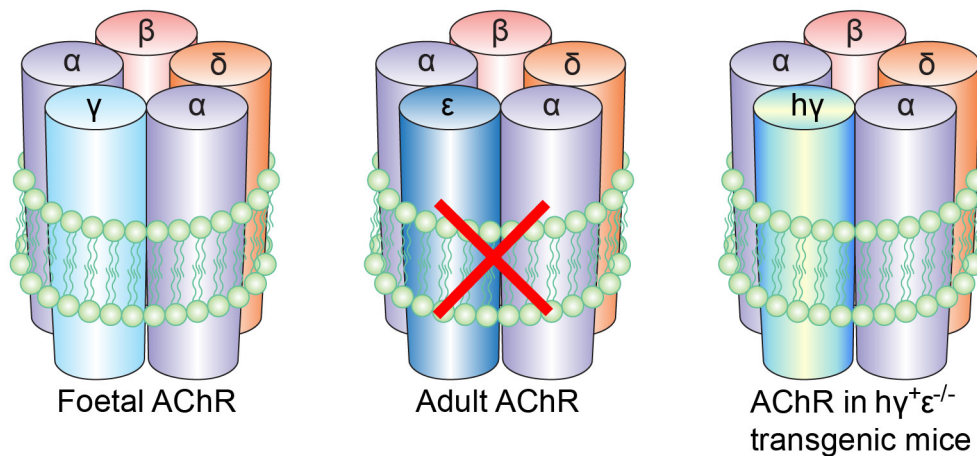


Figure 6-1 AChR expressed in $h\gamma^+\epsilon^{-/-}$ transgenic mice

The introduction of the human γ -subunit in mice with a *Chrne*-null background allows neuromuscular transmission to be mediated by the foetal $\alpha_2\beta\delta\epsilon$ AChR and $\alpha^2\beta\delta(h\gamma)$ AChR, and these mice survive in the absence of adult $\alpha_2\beta\delta\epsilon$ AChR. The $h\gamma^+\epsilon^{-/-}$ transgenic mice display features that are consistent with human AChR deficiency, including fatigable muscle weakness, reduced endplate AChR and aberrant neuromuscular transmission.

6.1.1 Analysis of endplate AChR clusters in the $hy^+ \epsilon^{-/-}$ mouse model of AChR deficiency syndrome

The structure of endplate AChR clusters in $hy^+ \epsilon^{-/-}$ mice appeared to be disrupted when compared to the pretzel-shaped AChR plaques in the $hy^+ \epsilon^{het}$ controls (Cossins et al., 2004). To quantitatively confirm this finding, a Fiji macro was developed to analyse the morphological differences. The protocol involves a background subtraction step that utilises a Gaussian blur filter, and subsequently analysis was performed on threshold-adjusted binary images of endplate AChR clusters (Figure 6-2).

The morphology of endplates on 4 different muscle groups: diaphragm, extensor digitorum longus (EDL), soleus and tibialis anterior (TA) muscle were analysed. Individual muscle groups were dissected and labelled with α -BuTx-594. Diaphragm muscles were then fixed and mounted; EDL, soleus and TA muscle fibres were teased prior to mounting to reveal NMJ for fluorescence microscopy. For each muscle group, 60 and 100 endplates, defined by α -BuTx-594-bound regions, from the $hy^+ \epsilon^{het}$ control (n=3) and $hy^+ \epsilon^{-/-}$ (n=5) mice were assessed respectively. (Figures 6-3 and 6-4)

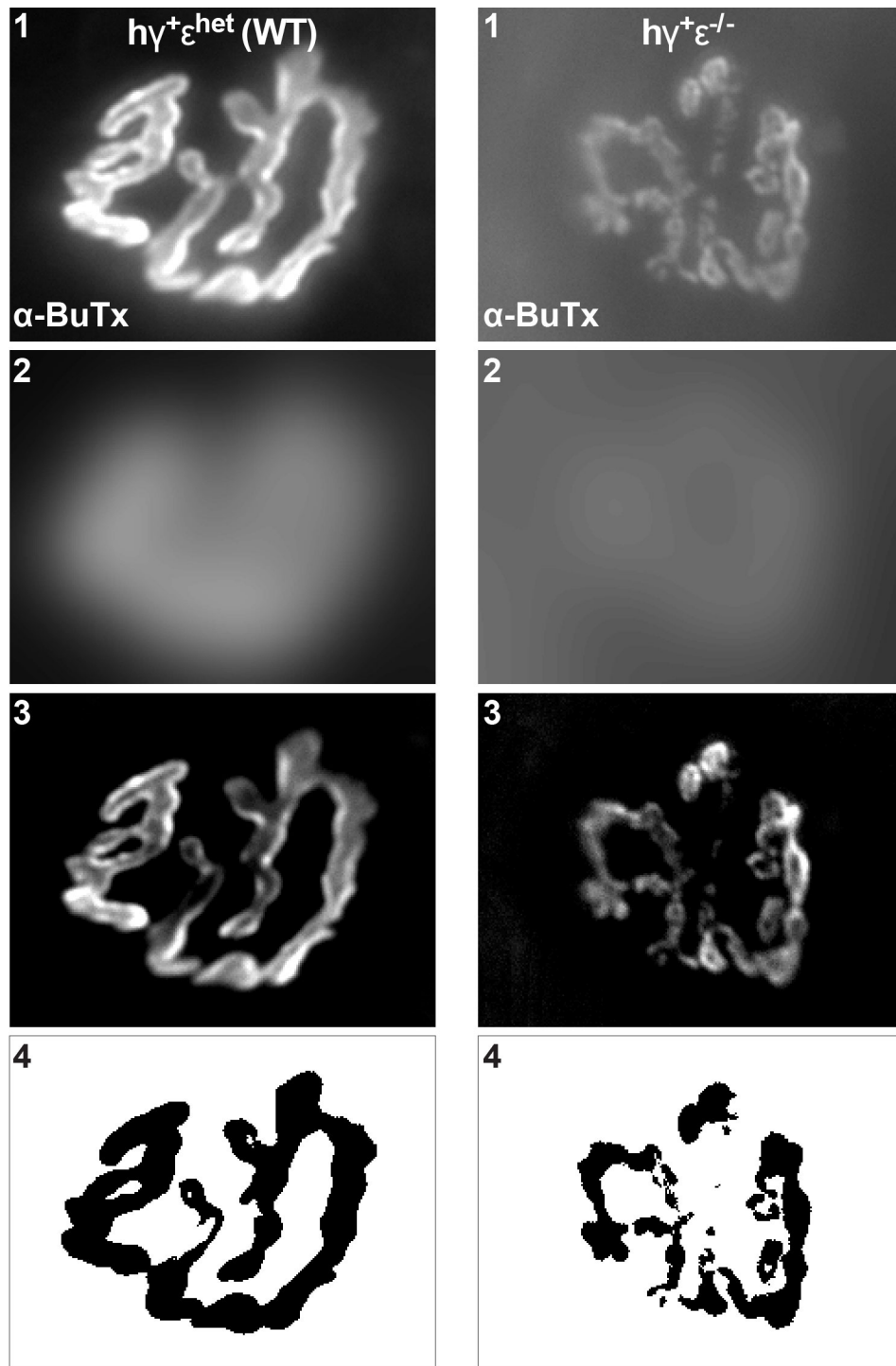


Figure 6-2 Method of quantifying endplate AChR clusters on muscle fibres

- (1) Endplate AChR from the EDL muscles of the $h\gamma^+\epsilon^{\text{het}}$ control and $h\gamma^+\epsilon^{-/-}$ mice were labelled with α -BuTx-594 and imaged by fluorescence microscopy at 40x magnification
- (2) A Gaussian blur filter was applied to the image to define background fluorescence
- (3) Background information on the filtered image (2) is subtracted from the original (1)
- (4) Isolation of endplate AChR by threshold adjustments to obtain a binary image for analysis

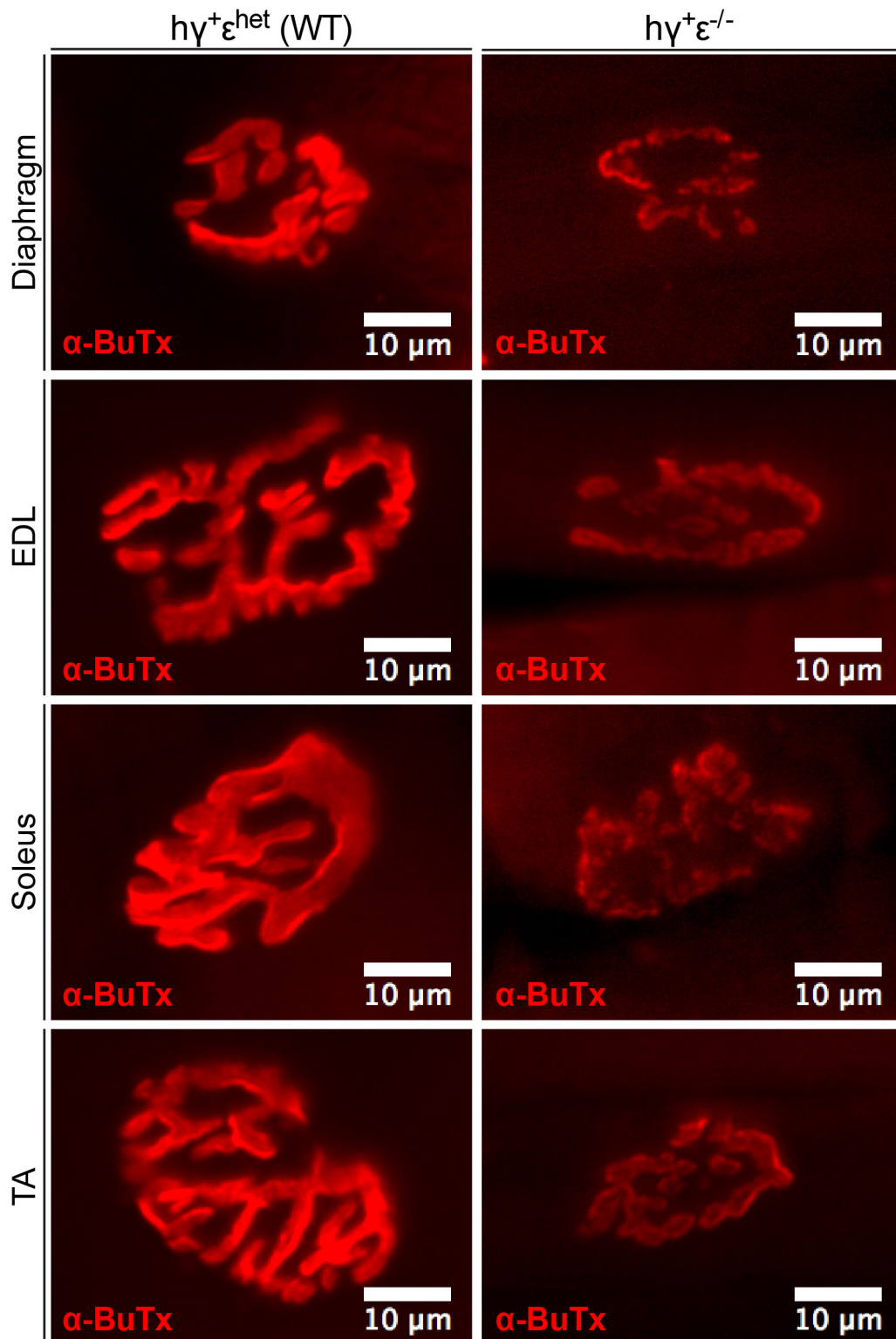


Figure 6-3 Representative images of endplate AChR clusters in $h\gamma^+\epsilon^{-/-}$ mice

Diaphragm, EDL, soleus and TA muscles were dissected from the $h\gamma^+\epsilon^{\text{het}}$ control and $h\gamma^+\epsilon^{-/-}$ mice, and AChR were labelled with α -BuTx-594. Intact diaphragm muscles and teased fibres from EDL, soleus and TA muscles were assessed by fluorescence microscopy. Images represent part of a microscopic field at 40x magnification.

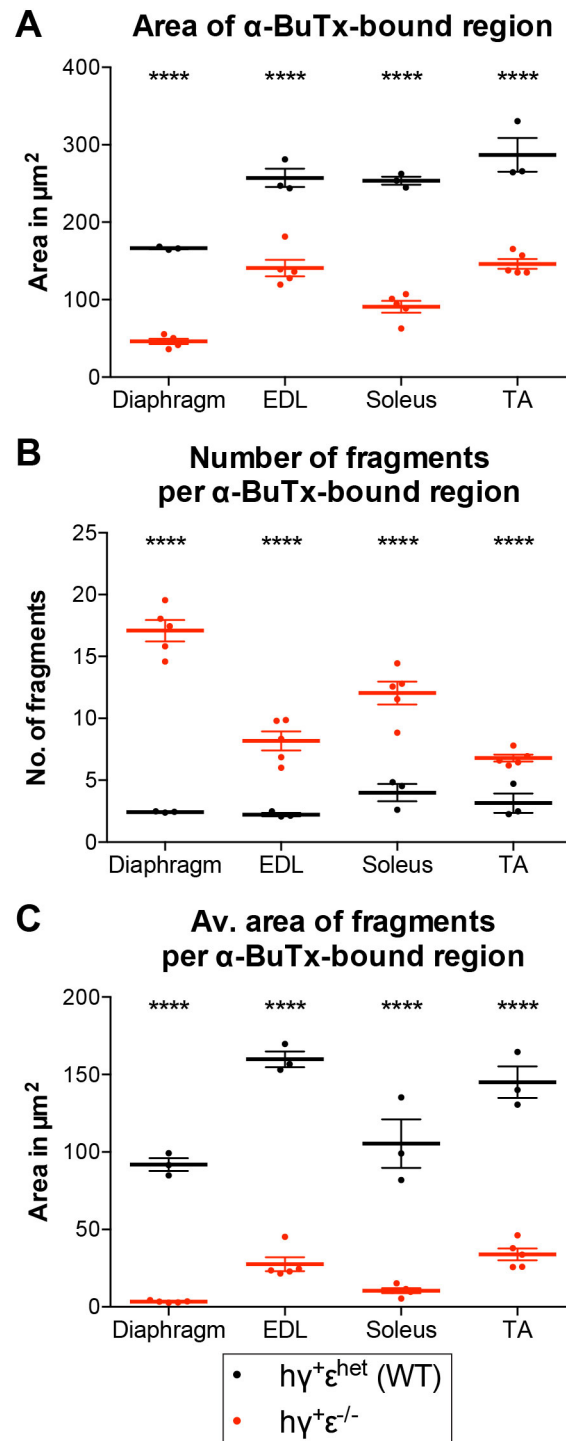


Figure 6-4 Quantified data on the morphology of endplate AChR clusters in $hy^+\epsilon^{-/-}$ mice

Diaphragm, EDL, soleus and TA muscles dissected from the $hy^+\epsilon^{het}$ control (n=3) and $hy^+\epsilon^{-/-}$ mice (n=5) were labelled with α -BuTx-594. Intact diaphragm muscles and teased fibres from EDL, soleus and TA muscles were analysed. Data points represent the mean of the data from 20 endplates and the results are summarised by mean $\pm$ SEM. Two-way ANOVA and Sidak's multiple comparisons test, asterisks ****p<0.0001

In all of the muscle groups analysed, the area of the α -BuTx-bound regions on $h\gamma^+\epsilon^{-/-}$ mice were significantly smaller compared to the $h\gamma^+\epsilon^{\text{het}}$ controls (Figure 6-4 **A**).

The number of fragments per α -BuTx-bound region were analysed to quantify the disrupted structure of the endplates on $h\gamma^+\epsilon^{-/-}$ muscles. In comparison to the $h\gamma^+\epsilon^{\text{het}}$ controls, there were significantly more fragments per α -BuTx-bound region on the muscles of $h\gamma^+\epsilon^{-/-}$ mice (Figure 6-4 **B**).

The average areas of the fragments of each α -BuTx-bound region on the muscles of $h\gamma^+\epsilon^{-/-}$ mice were found to be significantly smaller when compared to the $h\gamma^+\epsilon^{\text{het}}$ control mice (Figure 6-4 **C**).

Of note, the morphology of the endplates labelled with α -BuTx-594 differs between muscle groups in the $h\gamma^+\epsilon^{\text{het}}$ controls. Additional statistical analysis by One-way ANOVA and Dunnet's multiple comparison tests confirmed that the area of the α -BuTx-bound region on diaphragm muscles was significantly smaller than EDL, soleus and TA muscles ($p < 0.0001$ for all comparisons). Although there were no differences in the number of fragments per α -BuTx-bound region between muscle groups, the average size of the fragments on diaphragm and soleus muscles were significantly smaller than EDL ($p < 0.0001$ for both) and TA muscles ($p < 0.0001$ and $0 < 0.01$ respectively).

Hence the smaller and more fragmented endplate AChR clusters further confirmed the AChR deficiency phenotype of $h\gamma^+\epsilon^{-/-}$ mice.

6.1.2 Aims

Although most patients with AChR deficiency syndromes due to *RAPSN* or *CHRNE* mutations respond well to pyridostigmine and 3,4 DAP, some do not gain complete benefits from such treatments. Recently, 8 *CHRNE*-CMS and 1 *RAPSN*-CMS patients have been reported to benefit from the addition of β_2 -adrenergic receptor agonists to ongoing pyridostigmine treatment with or without 3,4-DAP (Rodríguez Cruz et al., 2015; Sadeh et al., 2011). However without data from a well-designed clinical trial, the use of salbutamol as a routine treatment for patients with AChR deficiency syndromes remains unlicensed. Moreover, the detailed molecular basis of how salbutamol benefits for CMS is currently not known. Hence an *in vivo* study may allow detailed molecular analysis to determine the mechanisms of salbutamol at the NMJ, and would provide a rational basis for incorporation into the treatment regimen for patients with AChR deficiency syndromes.

The aim of this chapter was to obtain preliminary data to evaluate if the addition of salbutamol to routine pyridostigmine treatment provides additional beneficial effects in a mouse model of AChR deficiency ($hy^+ \epsilon^{-/-}$). Data from this work serves as the basis to determine the experiment parameters for an expanded study that is now being carried out to determine the efficacy and mechanisms of salbutamol in $hy^+ \epsilon^{-/-}$ mice.

6.2 Experiment design

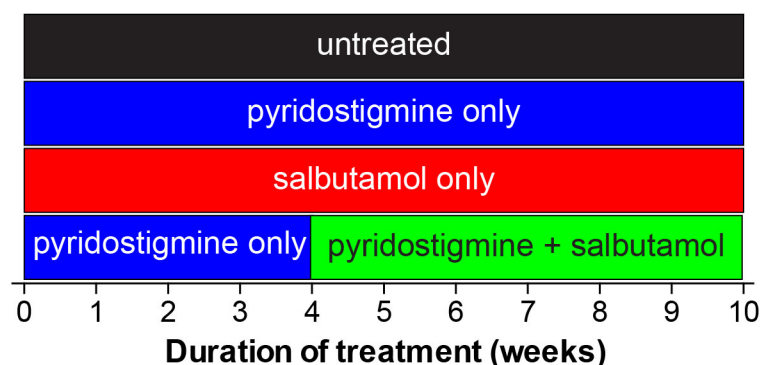


Figure 6-5 Experiment design to evaluate the efficacy of salbutamol treatment

For 10 weeks, the $hy^+ \epsilon^{-/-}$ mice were treated with pyridostigmine, salbutamol or the combination treatment with pyridostigmine for 4 weeks followed by pyridostigmine together with salbutamol until the end of the trial. Pyridostigmine and salbutamol were administered in the drinking water at 7mg/kg/day and 4.5mg/kg/day respectively.

Based on water consumption of 150ml/kg/day, pyridostigmine bromide (7mg/kg/day) or/and salbutamol sulphate (4.5mg/kg/day) was added to drinking water for $hy^+ \epsilon^{-/-}$ mice (aged 3-12 weeks at the start of treatments) for 10 weeks to evaluate their effects. Although studies have reported the beneficial effects of salbutamol in combination with pyridostigmine (Banwell et al., 2004; Rodríguez Cruz et al., 2015; Sadeh et al., 2011), it is not known if salbutamol alone offers some efficacy. Hence, the effects of 4 different regimens were tested: untreated (n=14), pyridostigmine only (n=13), salbutamol only (n=10) and pyridostigmine in combination with salbutamol (n=11). For the combination treatment, mice were treated with pyridostigmine for 4 weeks and salbutamol was added to the pyridostigmine solution for the following 6 weeks. Figure 6-5 summarises the treatment regimens used. To evaluate the effects of the different treatments, the growth, muscle strength and morphology of endplate AChR clusters in these mice were analysed.

6.3 Effects of treatments on growth

To evaluate the well-being of the $hy^+ \epsilon^{-/-}$ mice, the growth of all mice included in this study were assessed by weighing them twice on 2 separate days within the week prior to treatments, and once a week for the 10 week treatment period. The data for male (Figure 6-6) and female (Figure 6-7) mice were analysed separately, and presented as weights relative to the average of the baseline data before treatment.

6.3.1 Growth of male $hy^+ \epsilon^{-/-}$ mice

In the 4th weeks of treatment with pyridostigmine, male mice gained significantly more weight in comparison to untreated controls. This is evident in both groups of mice that received pyridostigmine only, and pyridostigmine with salbutamol. Except for mice that were given the combination treatment in week 5, the significant differences were maintained for both groups of mice until the end of the treatment period. By week 10, the relative weight gains of mice treated with pyridostigmine were ~20% higher than the untreated controls (Figure 6-6 **A**).

Salbutamol treatment alone did not alter the growth of male mice in comparison to untreated controls. When the weights of the mice treated with pyridostigmine only and pyridostigmine with salbutamol were normalised to their respective weights at week 4, the results confirmed that the addition of salbutamol to pyridostigmine treatment did not affect growth either. Hence the results suggested that the relative additional weight gain for male mice treated with pyridostigmine with salbutamol was due to the effects of pyridostigmine (Figure 6-6 **A** and **B**).

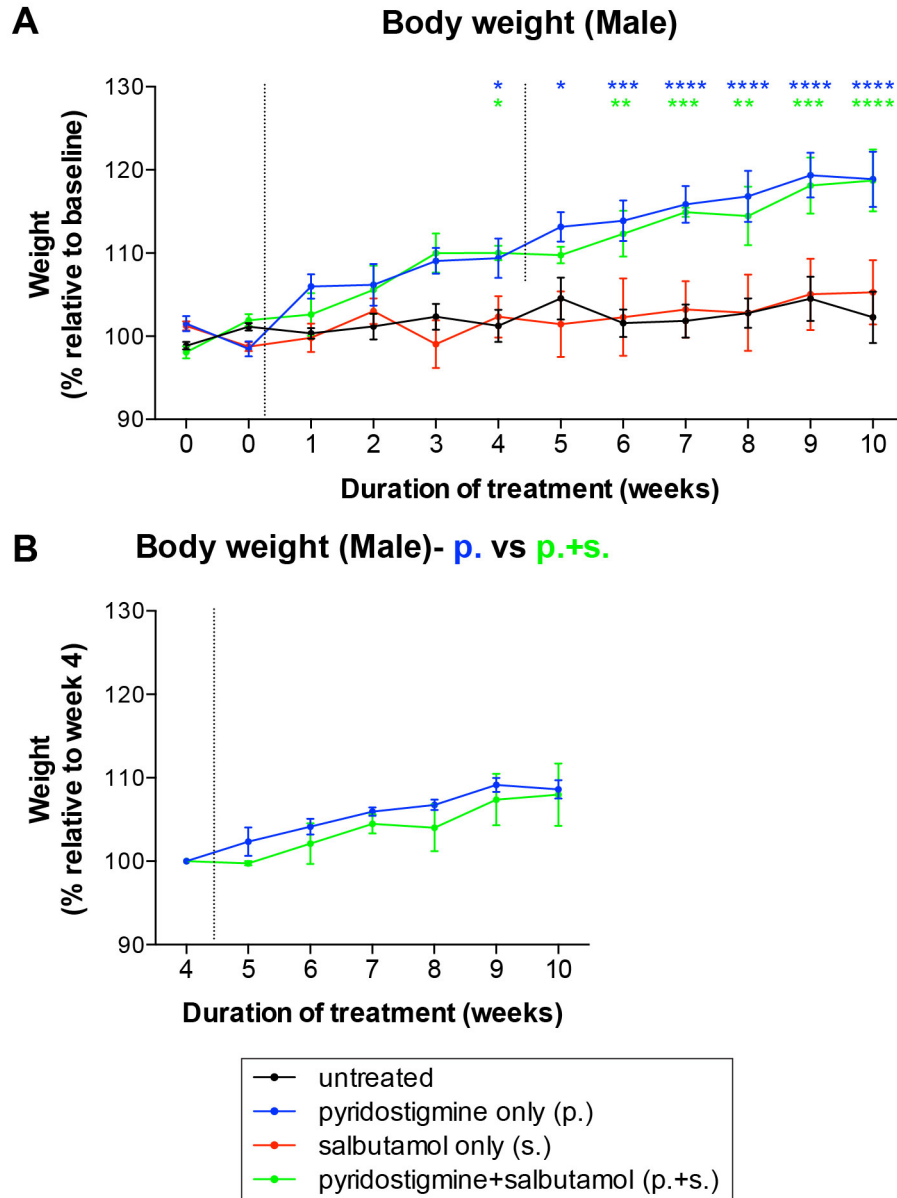


Figure 6-6 Growth of male $hy^+ \epsilon^{-/-}$ mice treated with pyridostigmine, salbutamol or a combination of both

The mice were weighed twice prior to the start of treatment (baseline, week 0), and then weighed once a week for the treatment period (10 weeks).

(A) The relative changes in weight from baseline at each time point are presented as mean percentages $\pm$ SEM for male $hy^+ \epsilon^{-/-}$ mice that were untreated (n=7), or treated with pyridostigmine (n=6), salbutamol (n=6) or both (n=5). The first dotted line represents the start of treatment, and the second represents the time when salbutamol was introduced to the combination treatment. Two-way ANOVA and Dunnett's multiple comparisons test, asterisks ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05.

(B) Weight changes relative to week 4 for mice treated with pyridostigmine alone or together with salbutamol. Two-way ANOVA and Sidak's multiple comparisons test.

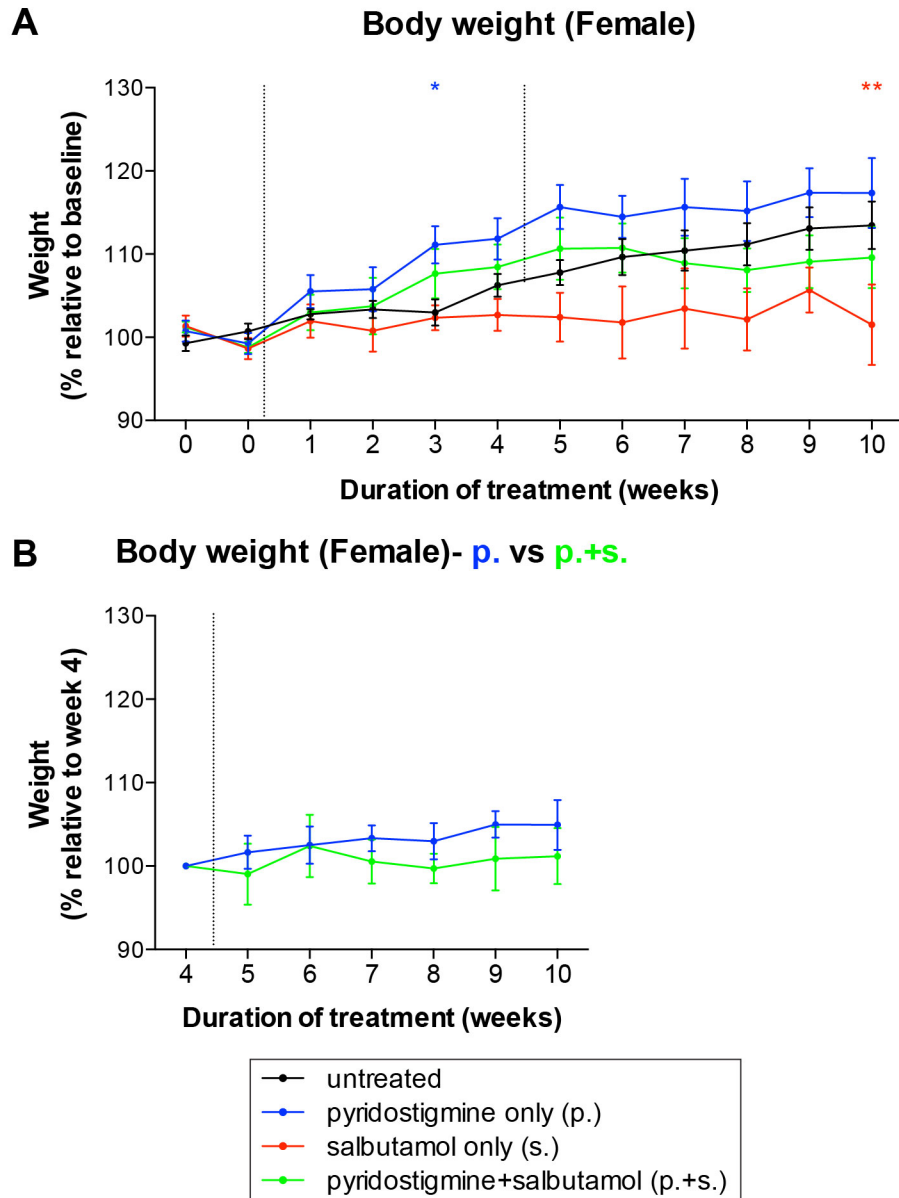


Figure 6-7 Growth of female $hy^{+}\epsilon^{-/-}$ mice treated with pyridostigmine, salbutamol or a combination of both

The mice were weighed twice prior to the start of treatment (baseline, week 0), and then weighed once a week for the treatment period (10 weeks).

(A) The relative changes in weight from baseline at each time point are presented as mean percentages $\pm$ SEM for female $hy^{+}\epsilon^{-/-}$ mice that were untreated ($n=7$), or treated with pyridostigmine ($n=7$), salbutamol ($n=4$) or both ($n=6$). The first dotted line represents the start of treatment, and the second represents the time when salbutamol was introduced to the combination treatment. Two-way ANOVA and Dunnett's multiple comparisons test, asterisks ** $p<0.01$, * $p<0.05$.

(B) Weight changes relative to week 4 for mice treated with pyridostigmine alone or together with salbutamol. Two-way ANOVA and Sidak's multiple comparisons test.

6.3.2 Growth of female $hy^+ \epsilon^{-/-}$ mice

The effect of pyridostigmine on female mice was less prominent. In the third week of treatment, pyridostigmine treated mice gained more weight when compared to untreated controls. Over the treatment period, although there was a trend of increased weight gain for mice treated with pyridostigmine only, statistical analysis suggested the relative weight gain for mice treated with pyridostigmine alone and pyridostigmine in combination with salbutamol were similar to untreated mice (Figure 6-7 A).

There was a trend for salbutamol to attenuate weight gain for female mice when compared to untreated mice, and a significant difference was detected at week 10. When the data of mice treated with pyridostigmine alone or in combination with salbutamol were normalised to their relative weights at week 4, the results show that the addition of salbutamol to pyridostigmine treatment did not alter the weight gain when compared to mice treated with pyridostigmine only (Figure 6.7 A, B).

6.3.3 Summary on the growth of $hy^+ \epsilon^{-/-}$ mice

In summary, male mice gained more weight after treatment with pyridostigmine, but salbutamol alone had no effect on their growth. On the other hand, the effect of pyridostigmine on growth for female mice was subtle, and salbutamol treatment alone reduced their relative weight gain in comparison to untreated controls. For both genders, the addition of salbutamol to pyridostigmine treatments did not alter the existing effects of pyridostigmine alone.

6.4 Effects of treatments on muscle strength

To evaluate the effects of treatments on fatiguable muscle weakness, the inverted screen test was performed on the mice twice before the start of treatments, and once a week during the 10 week treatment period. In the setup, $hy^{+}\epsilon^{-/-}$ mice were allowed to hang upside down from a wire screen until fatigued. The hanging times of mice from 3 continuous attempts were recorded and summed to represent their weekly performances. The data for male and female mice were analysed separately, and presented as hanging times relative to the baseline data for each treatment.

6.4.1 Muscle strength of male $hy^{+}\epsilon^{-/-}$ mice

In the first week after treatment started, the relative hanging time of male $hy^{+}\epsilon^{-/-}$ mice treated with pyridostigmine only significantly improved when compared to untreated controls. Since mice that were subjected to the combination treatment did not receive salbutamol until after week 4, their performances also improved in comparison to untreated controls due to the effects of pyridostigmine only. The significant improvements were maintained for both treatment groups for the 10 week period. Although not statistically significant, there is a trend for the performances of the mice in all of the treatment groups to decline from their maximum hanging times during the treatment period (Figure 6-8 A).

Treatment with salbutamol only did not affect the relative hanging times of male mice when compared to untreated controls for the entire treatment period. There was also no difference between the performances of mice that received pyridostigmine only and pyridostigmine with salbutamol (Figure 6-8 A and B).

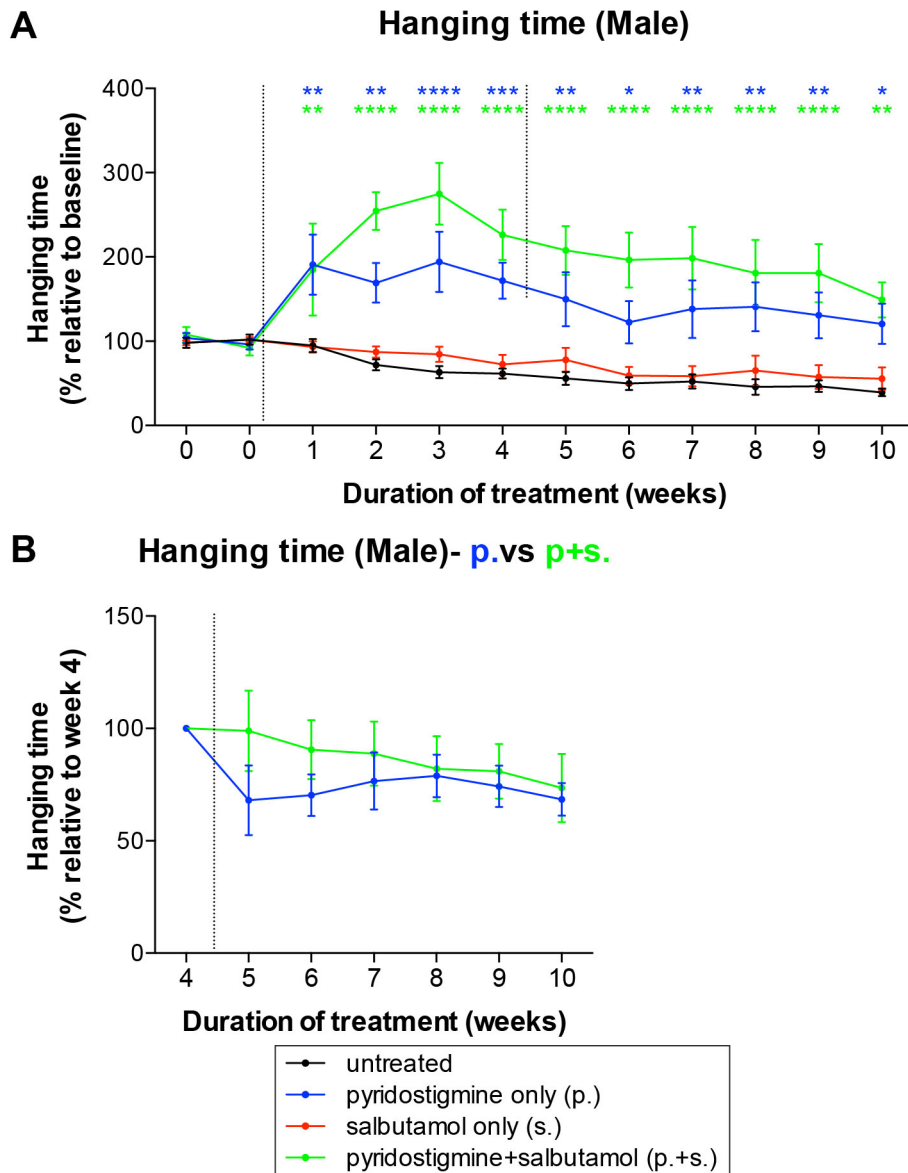


Figure 6-8 Inverted screen test performances of male $hy^+\epsilon^{-/-}$ mice treated with pyridostigmine, salbutamol or a combination of both

The muscle strength of mice was tested twice prior to the start of treatment (baseline, week 0), and then once a week for the treatment period (10 weeks).

(A) The relative performances (hanging time) from baseline at each time point are presented as mean percentages $\pm$ SEM for male $hy^+\epsilon^{-/-}$ mice that were untreated ($n=7$), or treated with pyridostigmine ($n=6$), salbutamol ($n=6$) or both ($n=5$). The first dotted line represents the start of treatments, and the second represents when salbutamol was introduced to the combination treatment. Two-way ANOVA and Dunnett's multiple comparisons test, asterisks **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$.

(B) Performances relative to week 4 for mice treated with pyridostigmine alone or together with salbutamol. Two-way ANOVA and Sidak's multiple comparisons test.

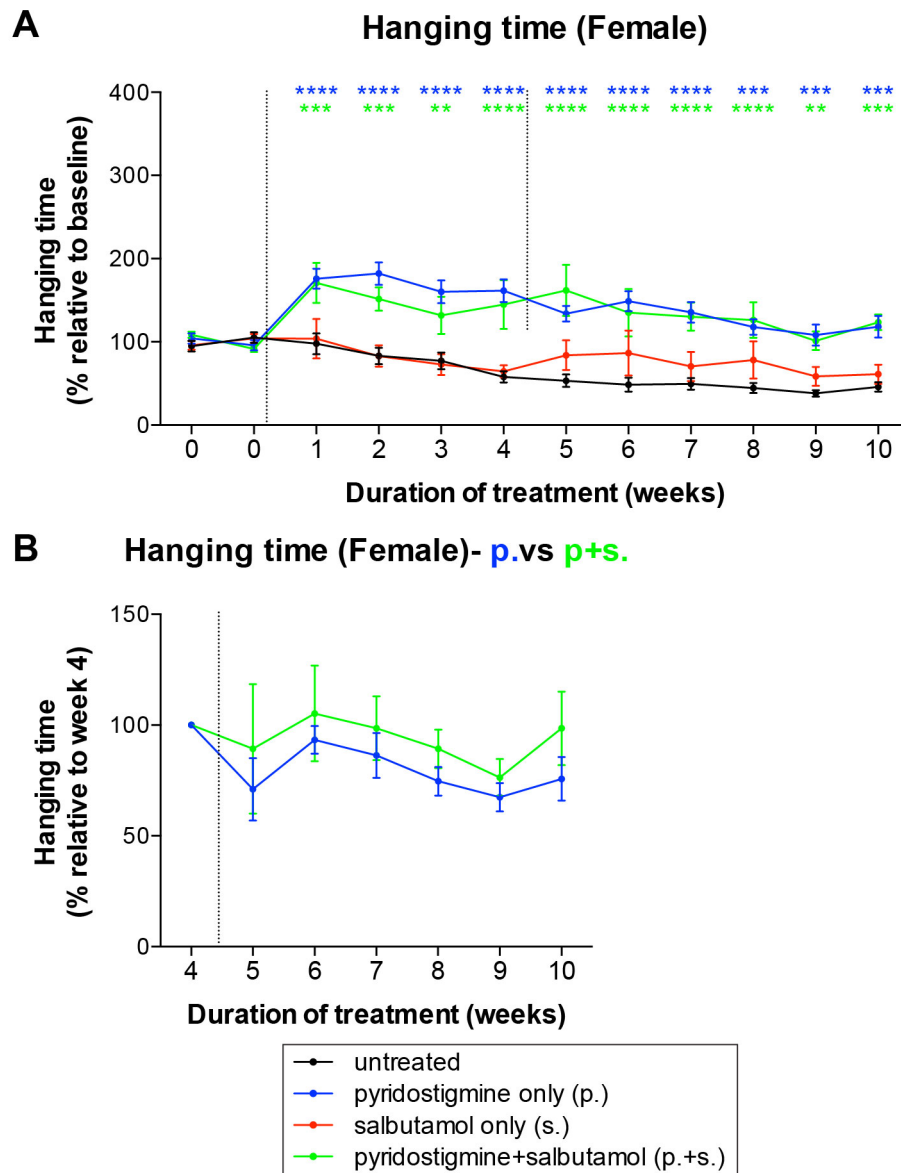


Figure 6-9 Inverted screen test performances of female $hy^+ \epsilon^{-/-}$ mice treated with pyridostigmine, salbutamol or a combination of both

The muscle strength of mice was tested twice prior to the start of treatment (baseline, week 0), and then once a week for the treatment period (10 weeks).

(A) The relative performances (hanging time) from baseline at each time point are presented as mean percentages $\pm$ SEM for female $hy^+ \epsilon^{-/-}$ mice that were untreated (n=7), or treated with pyridostigmine (n=7), salbutamol (n=4) or both (n=6). The first dotted line represents the start of treatments, and the second represents when salbutamol was introduced to the combination treatment. Two-way ANOVA and Dunnett's multiple comparisons test, asterisks ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05.

(B) Performances relative to week 4 for mice treated with pyridostigmine alone or together with salbutamol. Two-way ANOVA and Sidak's multiple comparisons test.

6.4.2 Muscle strength of female $hy^{+}\epsilon^{-/-}$ mice

The performances of female $hy^{+}\epsilon^{-/-}$ mice in the inverted screen test were very similar to those of male mice. In the first week of treatment, the relative hanging time of mice that were treated with pyridostigmine only was significantly improved in comparison to untreated controls. Since the data of weeks 1-4 of mice purposed for the combination treatment also represents pyridostigmine treated mice only, the significant improvement was also detected in this group of animals. The significant improvements in comparison to untreated mice were maintained for the duration of the treatment period. Again, there was a noticeable trend for the performances to decline over the treatment period (Figure 6-9 A).

Salbutamol had no significant effect on the hanging time of female $hy^{+}\epsilon^{-/-}$ mice in comparison to untreated mice. Comparisons made between mice that received pyridostigmine only and pyridostigmine with salbutamol after week 4 revealed no difference in their performances either (Figure 6-9 A and B).

6.4.3 Summary on the muscle strength of $hy^{+}\epsilon^{-/-}$ mice

In summary, pyridostigmine significantly improved the muscle strengths of both male and female $hy^{+}\epsilon^{-/-}$ mice within less than a week of treatment, and the improvements lasted for the duration of the 10 week period. Salbutamol alone or in combination with pyridostigmine did not affect the performances of $hy^{+}\epsilon^{-/-}$ mice in the inverted screen test. Although not statistically significant, the noticeable trend of reduced hanging times of all animals suggests that none of the treatments tested were able to counteract the trend of progressive worsening in muscle weakness.

6.5 Sub-optimal serum salbutamol concentration

Due to unforeseeable circumstances, the serum concentration of salbutamol could not be analysed before this pilot study was undertaken. The serum salbutamol concentrations of $h\gamma^+\epsilon^{\text{het}}$ mice caged together with salbutamol treated $h\gamma^+\epsilon^{-/-}$ mice were determined after the 10 week treatment period by Dr. James Wickens at the Department of Chemistry. Disappointingly, the sera contained 1.253ng/ml $\pm$ 0.1578 of salbutamol (n=5), which is much lower than anticipated.

According to the dose translation based on body surface area (Reagan-Shaw et al., 2008), the dose of salbutamol used for the mice, 4.5mg/kg/day, translates to 0.36mg/kg/day for the human adult estimated dose. From the recent report, 8mg/day of oral salbutamol was found to benefit AChR deficiency patients that were aged between 14-53 years old at the time of treatment (Rodríguez Cruz et al., 2015). If the human estimated dose of 0.36mg/kg/day was applied to a 60kg human subject, 21.6mg/day would be required, which is significantly higher than the dose currently used for AChR deficiency patients. In humans, 20mg/day oral salbutamol consumption for 2 weeks leads to a serum salbutamol concentration of ~8-12ng/ml (Haukkamaa et al., 1985). The sera salbutamol concentration of the $h\gamma^+\epsilon^{-/-}$ mice was almost 10-fold less than expected.

In summary, the low serum salbutamol concentration in $h\gamma^+\epsilon^{\text{het}}$ mice suggests that the dose of salbutamol consumed by $h\gamma^+\epsilon^{-/-}$ mice is sub-optimal. Any efficacy of salbutamol for this animal model is unlikely to be reflected in this pilot study.

6.6 Effects of treatments on the morphology of endplate AChR clusters

After the 10 weeks of treatment with pyridostigmine only (n=2), salbutamol only (n=5) or a combination of both (n=3), the diaphragm, EDL, soleus and TA muscles were dissected from the $h\gamma^+\epsilon^{-/-}$ mice and untreated controls (n=5) for analysis of the morphology of endplate AChR labelled with α -BuTx-594. For each muscle group of each $h\gamma^+\epsilon^{-/-}$ mouse, 20 α -BuTx-bound regions were analysed (Figures 6-10 and 6-11).

There were no obvious changes to the morphology of endplate AChR after the mice were treated with any of the treatment regimens. With 1 exception, the quantified results detected no significant differences caused by the treatments to the area of α -BuTx-bound regions, fragmentation and average area of fragments per α -BuTx-bound region on any of the muscles.

Pyridostigmine in combination with salbutamol treatment significantly increased the fragmentation of endplates on the soleus muscle of $h\gamma^+\epsilon^{-/-}$ mice, but not the other muscle groups analysed.

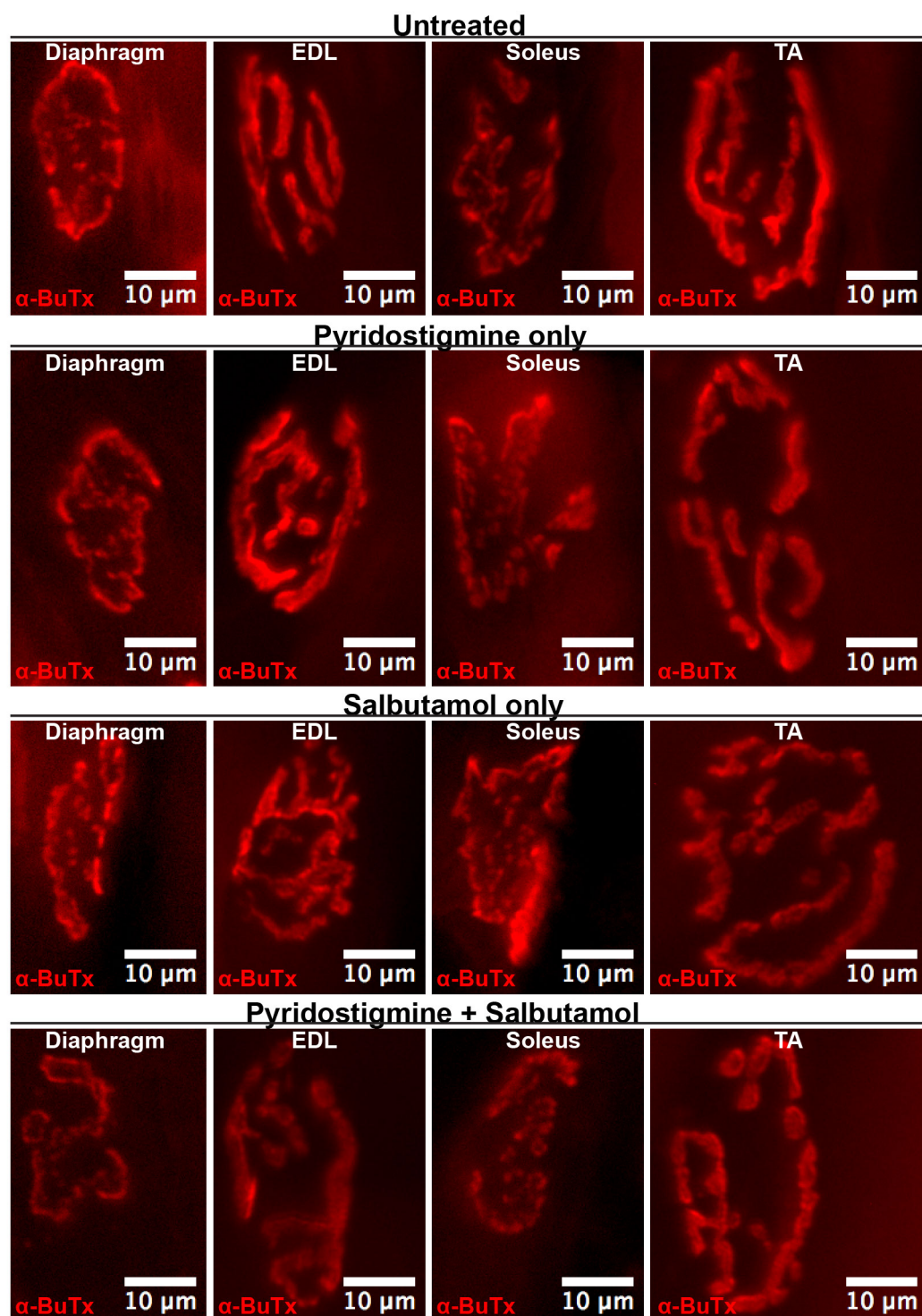


Figure 6-10 Endplate AChR clusters of $hy^{+}\epsilon^{-/-}$ mice treated with pyridostigmine, salbutamol or a combination of both

Diaphragm, EDL, soleus and TA muscles were dissected from $hy^{+}\epsilon^{-/-}$ mice that were untreated or treated with pyridostigmine, salbutamol or a combination of both for 10 weeks, and AChR clusters were labelled with α -BuTx-594. Intact diaphragm muscles and teased fibres from EDL, soleus and TA muscles were assessed by fluorescence microscopy. Images represent part of a microscopic field at 40x magnification.

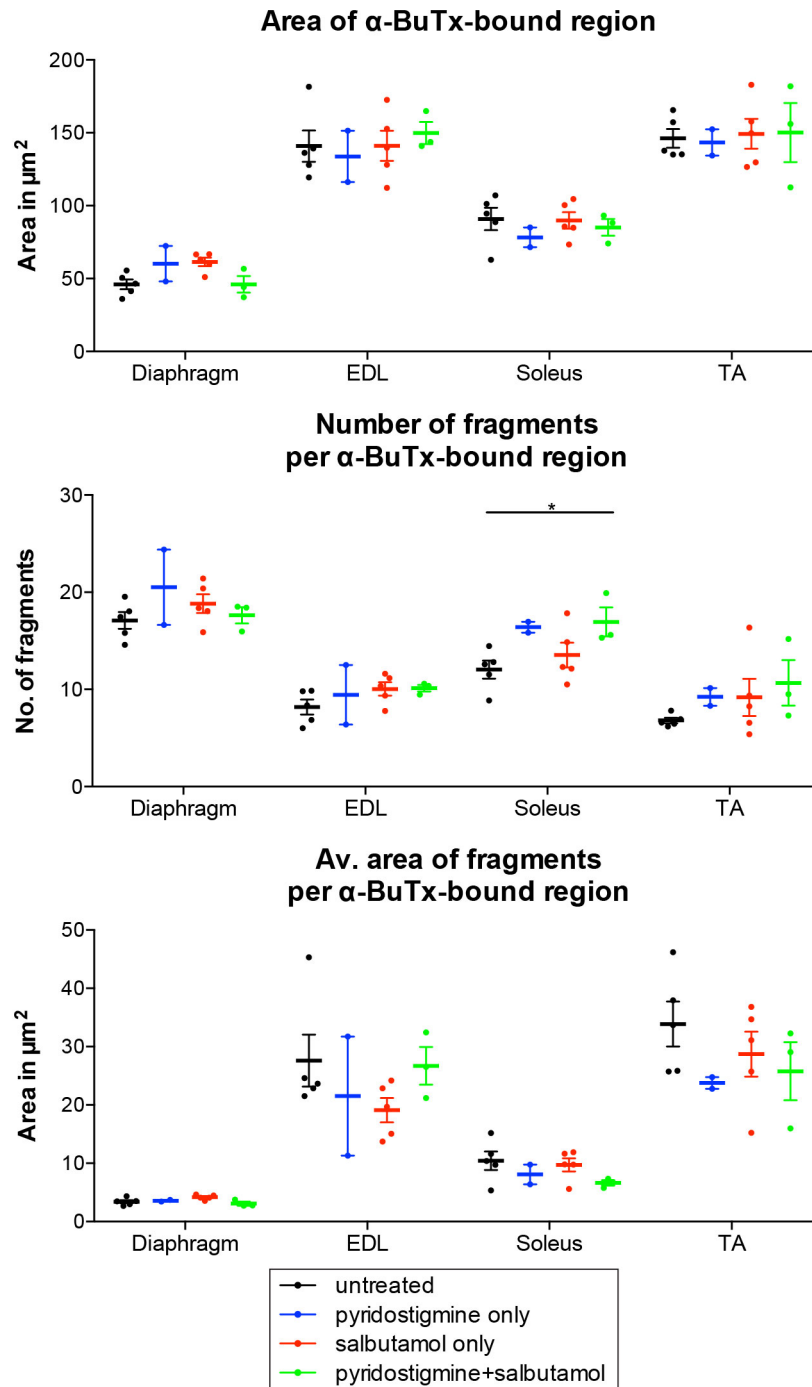


Figure 6-11 Quantified data on the morphology of endplate AChR clusters of $hy^+\epsilon^{-/-}$ mice treated with pyridostigmine, salbutamol or a combination of both

Diaphragm, EDL, soleus and TA muscles from $hy^+\epsilon^{-/-}$ mice, which were untreated (n=5) or treated with pyridostigmine only (n=2), salbutamol only (n=5) or a combination of both (n=3) for 10 weeks, were dissected. Subsequently endplate AChR were labelled with α -BuTx-594 and analysed.

Data points represent the mean of data from 20 endplates, and are summarised by mean $\pm$ SEM. Statistical confirmations were obtained by One-way ANOVA and Dunnett's multiple comparisons tests for each muscle group, asterisk *p<0.05

6.7 Discussion

The chapter aimed to preliminarily evaluate the efficacy of salbutamol treatments in the $hy^+ \epsilon^{-/-}$ mouse model of AChR deficiency syndrome. Due to the unexpected low serum salbutamol concentrations, the presented data on the effects of salbutamol treatments is not comparable to reports on its use in CMS-patients. It is necessary to optimise the current treatment regimens to obtain reliable insights. Nonetheless pyridostigmine treatments in the animal model reaffirmed its ability to improve muscle strength. An additional finding was that pyridostigmine treatments increased the growth of male animals. Finally, the analyses on the morphologies of endplate AChR clusters suggest that improvements in muscle strength occur without obvious alterations to the structure of endplate AChR clusters (summarised in Table 6-1).

Table 6-1 Summary of the effects of drug treatments on $hy^+ \epsilon^{-/-}$ mice

Effects of treatments (in comparison to untreated controls)				
		Pyridostigmine only	Salbutamol only	Pyridostigmine + Salbutamol
Growth	Male	Increased	=	Increased
	Female	=	Reduced	=
Muscle strength	Male	Increased	=	Increased
	Female	Increased	=	Increased
*Area of endplate AChR	Diaphragm	=	=	=
	EDL	=	=	=
	Soleus	=	=	=
	TA	=	=	=
	Diaphragm	=	=	=
*Fragmentation of endplate AChR	EDL	=	=	=
	Soleus	=	=	Increased
	TA	=	=	=
	Diaphragm	=	=	=
*Average area of fragments per endplate AChR	EDL	=	=	=
	Soleus	=	=	=
	TA	=	=	=

Due to the lower than expected serum concentration of salbutamol in the treated mice, the effects of salbutamol may not be truly reflected in this study.

*Only 2 $hy^+ \epsilon^{-/-}$ mice treated with pyridostigmine only were analysed. Thus the data requires further confirmation.

6.7.1 Dosages of pyridostigmine and salbutamol

According to calculations, the 4.5mg/kg/day dose of salbutamol should have provided a level higher than the effective dose for humans. Salbutamol was delivered to mice in their drinking water, which was refreshed once a week. The half-life of salbutamol in a solution of pH7.1 is ~11 days (Mälkki and Tammilehto, 1990). The sub-optimal dose received by the mice is unlikely to be due to degradation of salbutamol in the solution. It may be that the current estimated effective dose used was simply ineffective. 7mg/kg/day of pyridostigmine was previously established in our laboratory to be an effective dose to improve the muscle strengths of $hy^{+/-}$ mice. If 7mg/kg/day was calculated by the formula for dose translation based on body surface area (Reagan-Shaw et al., 2008), 58mg/kg/day and 0.84mg/kg/day would have been suggested as the mouse and human equivalent dose respectively. Administration of pyridostigmine at 25mg/kg/day to healthy rats can cause death (Richtsfield et al., 2013). It is clearly better to measure serum drug concentrations, but there were delays in achieving this, which were beyond our laboratory's direct control. Effective titration of dosing will be required before a full experimental investigation.

Salbutamol treatment at 8mg/kg/day, delivered by an Alzet minipump, was reported to improve muscle strength in a mouse model of anti-MuSK myasthenia gravis (Ghazanfari et al., 2014). However, the serum levels of salbutamol of the treated mice were not reported. It has been demonstrated that intravenous administration of salbutamol in humans leads to double the serum concentration of salbutamol than when given orally (Haukkamaa et al., 1985). A potential method to optimise the dose could be via the use of an Alzet minipump.

Although salbutamol was seen to have a positive beneficial effect in a model of anti-MuSK myasthenia gravis (Ghazanfari et al., 2014), it has not yet been established if salbutamol works through the same pathway in mice and in humans.

6.7.2 Effects of treatments on growth of $hy^+ \epsilon^{-/-}$ mice

Although the dosage of salbutamol was found to be suboptimal, female mice that were treated with salbutamol alone gained significantly less weight compared to untreated controls. This finding is in contrast with the report that found salbutamol reduced weight loss in female mice in an anti-MuSK myasthenia gravis model (Ghazanfari et al., 2014). It is noteworthy that the salbutamol treated female mice had a similar relative increase in weight to male mice that untreated or treated with salbutamol. The impact of salbutamol treatment on the growth of $hy^+ \epsilon^{-/-}$ mice will need further confirmation.

The higher growth was seen in male mice treated with pyridostigmine only. Although less prominent, the same trend in higher growth was observed in female mice. An explanation for the increased in growth is that improved muscle strength of the mice leads to higher mobility and more food consumption.

6.7.3 Effects of treatments on muscle strength of $hy^{+}\epsilon^{-/-}$ mice

The results show that the current dose of salbutamol was ineffective at providing any functional improvements in muscle strength. Pyridostigmine improved the muscle strengths of $hy^{+}\epsilon^{-/-}$ mice days after the treatment was started, which agrees with the observations in patients that it is effective as soon as it has been absorbed (Engel et al., 2015), and in mice that acetylcholinesterase activities changed within hours after treatment (Lintern et al., 1997). However, the current dose did not provide full recovery of muscle strength to levels of the $hy^{+}\epsilon^{het}$ controls (data not shown). Most AChR deficiency patients only gain partial benefits from pyridostigmine (Engel, 2007; Sadeh et al., 2011). Further titration of the treatment dose for $hy^{+}\epsilon^{-/-}$ mice may lead to further improvements in the muscle strengths of the mice. 16mg/kg/day of pyridostigmine did not show signs of toxicity in mice (Morsch et al., 2013).

Although the significant differences in untreated controls were maintained, the improved hanging times of pyridostigmine treated mice were not sustained (week1: 459.5seconds SEM $\pm$ 56.02; performance in week10: 299.0seconds SEM $\pm$ 39.32). Equival observations may be seen in humans (Rodríguez Cruz et al., 2015), as high dosage or prolonged use of acetylcholinesterase inhibitors can cause counteractive side-effects (Gold et al., 2008). Excitotoxicity due to excessive acetylcholine stimulation can lead to the overloading of cation at the postsynaptic membrane and subsequent endplate remodelling and muscle weaknesses (Deflorio et al., 2014; Engel, 2012; Gold et al., 2008).

6.7.4 Effects of treatments on the morphology of endplate AChR clusters of $\text{hy}^+\epsilon^{-/-}$ mice

In addition to the reduced size of endplate AChR clusters, fragmentation of the AChR clusters is also a feature in the $\text{hy}^+\epsilon^{-/-}$ mouse model of AChR deficiency. Similar morphological changes at the NMJ have been reported from a mouse model of anti-MuSK myasthenia gravis (Ghazanfari et al., 2014) and transgenic mice that overexpressed the neuronal protease neurotrypsin, which rendered agrin inactive (Butikofer et al., 2011). The agrin/MuSK signalling cascade is important for both the assembly and stabilisation of AChR clusters (Bezakova et al., 2001; Brockhausen et al., 2008). The stability of AChR endplates may also be affected in $\text{hy}^+\epsilon^{-/-}$ mice. The mouse foetal $\alpha_2\beta\delta\gamma$ and adult $\alpha_2\beta\delta\epsilon$ forms of AChR had similar half-lives when expressed in heterologous cells (Liu et al., 1994). However, the stability of endplate $\alpha_2\beta\delta\text{hy}$ receptors that are present in this animal model has not been investigated.

Although treatment with pyridostigmine significantly improved muscle strength, the morphology of endplate AChR clusters on the diaphragm, EDL, and TA muscles of $\text{hy}^+\epsilon^{-/-}$ mice that were treated with pyridostigmine were not improved. The morphology of endplates on the soleus muscle of mice that were given pyridostigmine in combination with salbutamol for 10 weeks were significantly more fragmented. Of note, only 2 mice that were treated with pyridostigmine only were analysed for the morphology of endplates. Thus, that the precise treatment regimen increased the fragmentation of endplate AChR clusters in the soleus muscle requires further confirmation. The results suggest that different muscle groups may be differentially affected by the treatments (Lintern et al., 1997), and the detected fragmentation of endplate AChR clusters on the soleus muscle is subclinical.

It is probable for the subclinical detrimental effects on the NMJ in the soleus muscle to derive from the chronic administration of pyridostigmine. In rats, long-term anticholinesterase therapy has adverse effects on the synaptic structure and neuromuscular transmission (Engel et al., 1973). A study reported that a high dose of pyridostigmine treatment (25mg/kg/day) for 28 days resulted in downregulation in the number of AChR in rats (Richtsfield et al., 2013).

Pyridostigmine acts to inhibit acetylcholinesterase and increase synaptic acetylcholine concentrations. Since acetylcholine is involved in the disassembly of AChR clusters (Lin et al., 2005; Misgeld et al., 2005), the prolonged agonist stimulation of AChR in the $hy^{+}\epsilon^{-/-}$ mice could lead to the activation of the calpain/Cdk5 pathway and result in the fragmentation of AChR clusters. Endplate acetylcholinesterase deficiency is another subtype of CMS that results in fatiguable muscle weaknesses (Hutchinson et al., 1993), and prolonged anticholinesterase medication could be acting through a similar pathway.

The possible sub-optimal dose of salbutamol and the heterogeneity of endplate morphology between individual $hy^{+}\epsilon^{-/-}$ mice hindered definitive investigations into the effects of salbutamol on endplate morphology. In the mouse model of anti-MuSK myasthenia gravis, salbutamol treatment alone reduced the degree of fragmentation of endplates AChR clusters (Ghazanfari et al., 2014). It would be of interest to determine if salbutamol, at an optimal dose, can provide similar benefits for the endplates of $hy^{+}\epsilon^{-/-}$ mice.

6.7.5 Potential methods of analysis of endplate morphology

The current protocol for analysis of endplate AChR clusters could be optimised for sensitivity and retrievable information. The use of a second NMJ marker, fasciculin 2-488, in combination with α -BuTx-594 may provide information on the endplate AChR in relation to the size/structure of the NMJ. Fasciculin 2 is a toxin that binds to acetylcholinesterase in the synaptic space. Thus the suggested analysis of the morphology of endplates may also suggest whether pyridostigmine treatments alter acetylcholinesterase expression at levels that are detectable by microscopy.

The sensitivity of the analysis could be improved if performed on maximum intensity projection confocal stack images that were taken at higher magnification power. (Figures 6-12, 6-13, 6-14 and 6-15 shows examples confocal images of α -BuTx-594 and fasciculin 2-488 labelling) To detect subtle changes in the degree of fragmentation of AChR clusters, 3-dimensional analysis could be performed on the stack images by Imarus software. Of note, to varying degrees, the signals detected from the α -BuTx and fasciculin 2 fluorescent conjugates by a confocal microscope revealed structures of postsynaptic folds on the muscles of $hy^+ \epsilon^{het}$ control mice, which is less apparent on the fragmented AChR clusters in $hy^+ \epsilon^{-/-}$ mice.

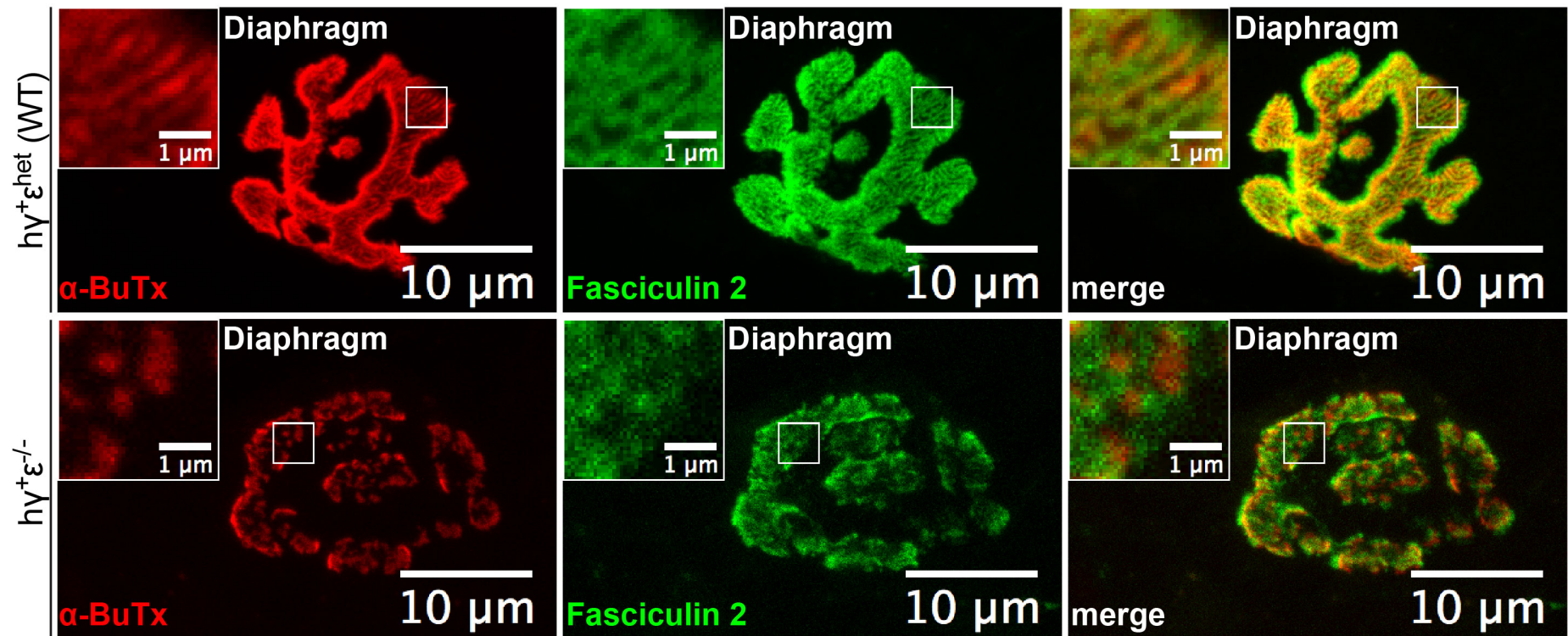


Figure 6-12 Maximum intensity projection confocal images of endplates on diaphragm muscles of $hy^+ \epsilon^{het}$ and $hy^+ \epsilon^{-/-}$ mice

Diaphragm muscles were dissected from $hy^+ \epsilon^{het}$ control and $hy^+ \epsilon^{-/-}$ mice, and labelled with α -BuTx-594 and fasciculin 2-488. Intact diaphragm muscles were mounted and assessed by confocal microscopy.

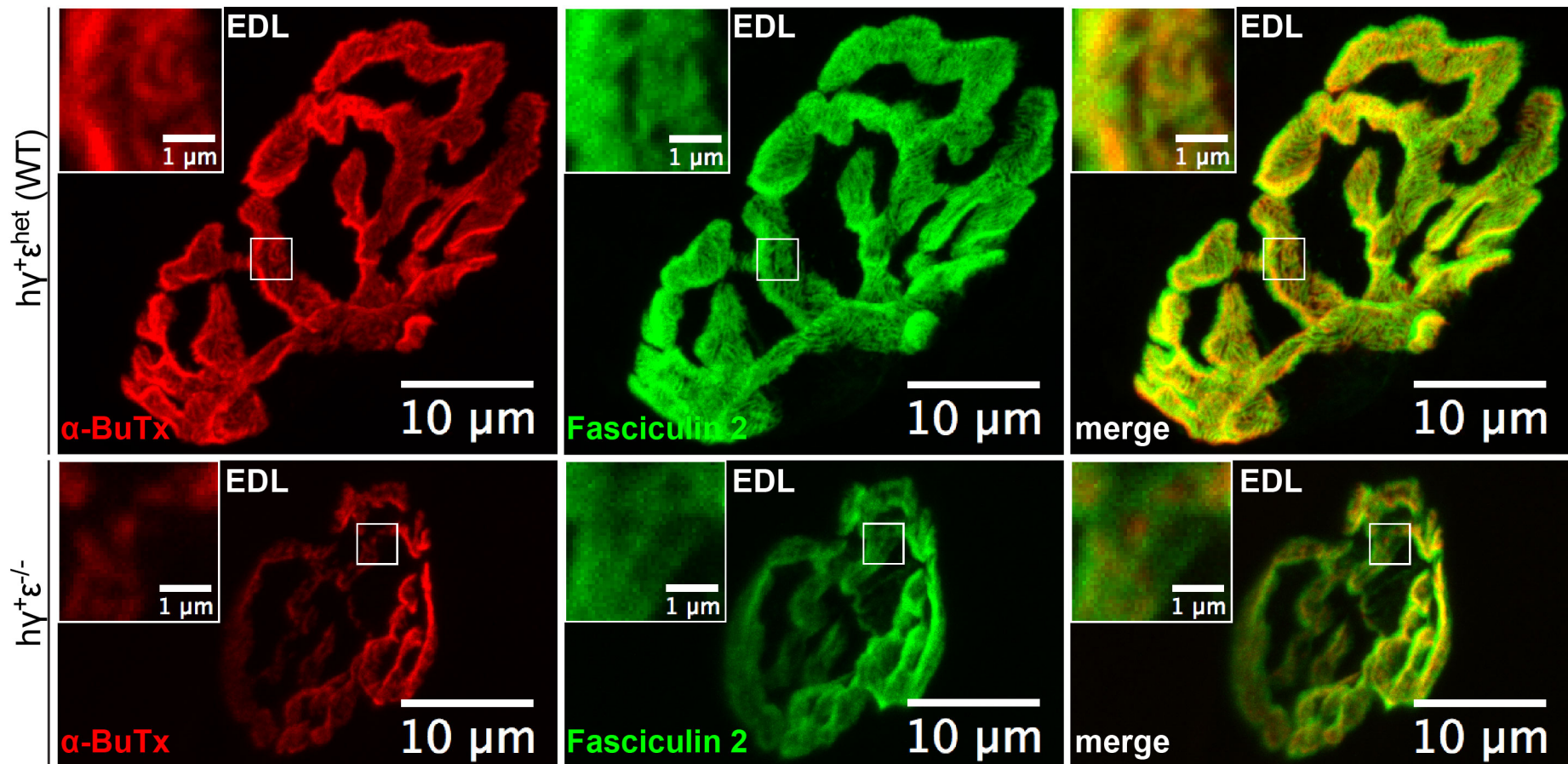


Figure 6-13 Maximum intensity projection confocal images of endplates on EDL muscles of $hy^{+}\epsilon^{het}$ and $hy^{+}\epsilon^{-/-}$ mice

EDL muscles were dissected from $hy^{+}\epsilon^{het}$ control and $hy^{+}\epsilon^{-/-}$ mice, and labelled with α -BuTx-594 and fasciculin 2-488. Teased muscle fibres were assessed by confocal microscopy.

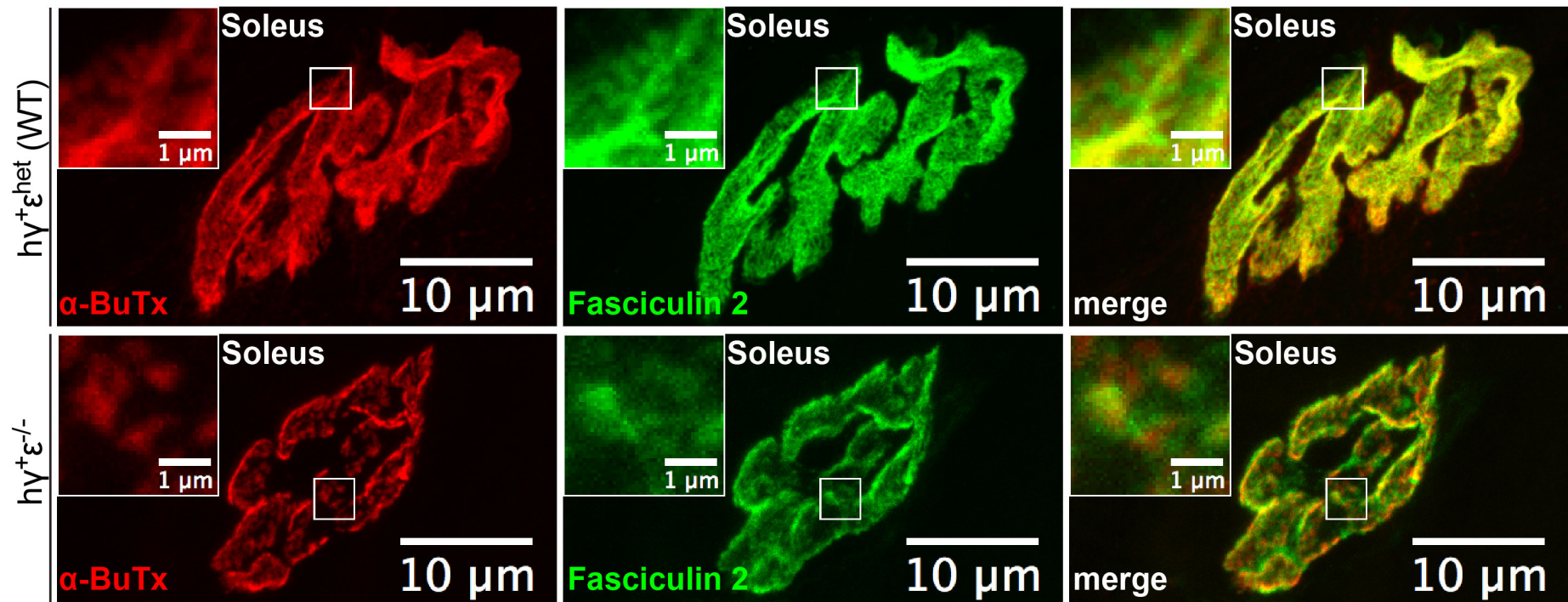


Figure 6-14 Maximum intensity projection confocal images of endplates on soleus muscles of $h\gamma^+\epsilon^{het}$ and $h\gamma^+\epsilon^{-/-}$ mice

Soleus muscles were dissected from $h\gamma^+\epsilon^{het}$ control and $h\gamma^+\epsilon^{-/-}$ mice, and labelled with α -BuTx-594 and fasciculin 2-488. Teased muscle fibres were assessed by confocal microscopy.

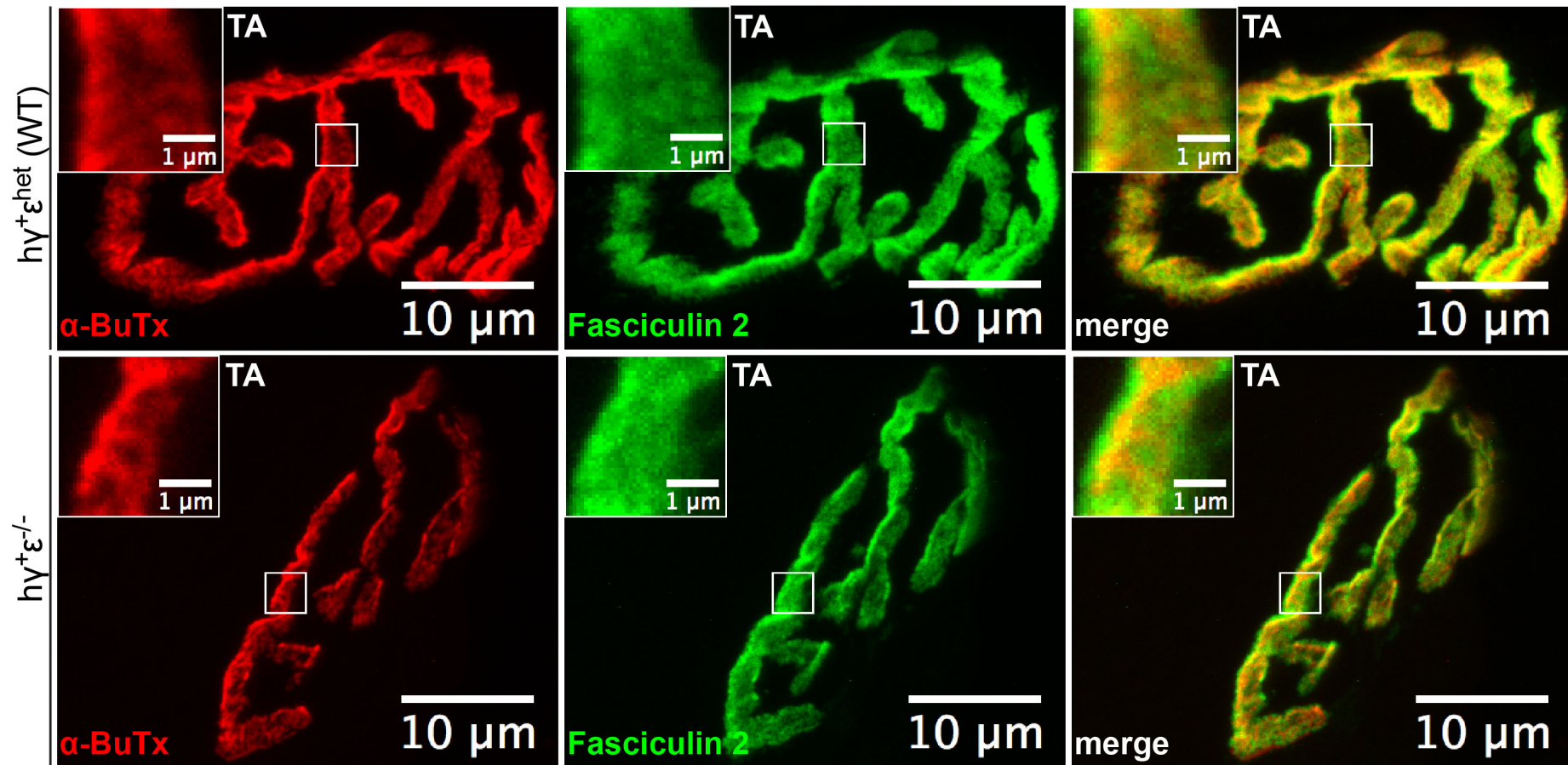


Figure 6-15 Maximum intensity projection confocal images of endplates on TA muscles of $hy^+ \epsilon^{het}$ and $hy^+ \epsilon^{-/-}$ mice

TA muscles were dissected from $hy^+ \epsilon^{het}$ control and $hy^+ \epsilon^{-/-}$ mice, and labelled with α -BuTx-594 and fasciculin 2-488. Teased muscle fibres were assessed by confocal microscopy.

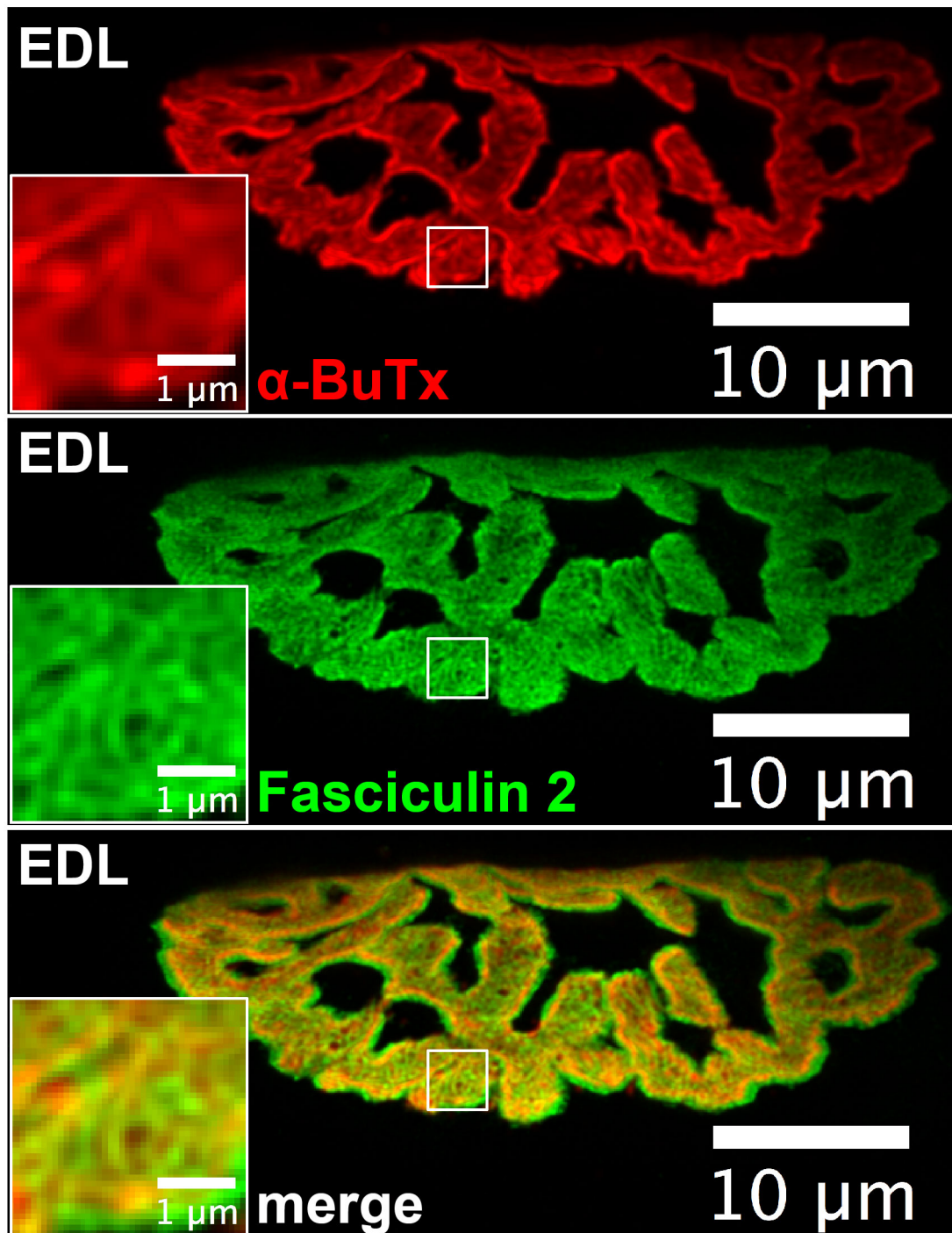


Figure 6-16 Maximum intensity projection confocal image of an endplate on EDL muscle of $hy^+ \epsilon^{het}$ mice

The EDL muscle was dissected from $hy^+ \epsilon^{het}$ control mice, and labelled with α -BuTx-594 and fasciculin 2-488. Teased muscle fibres were assessed by confocal microscopy.

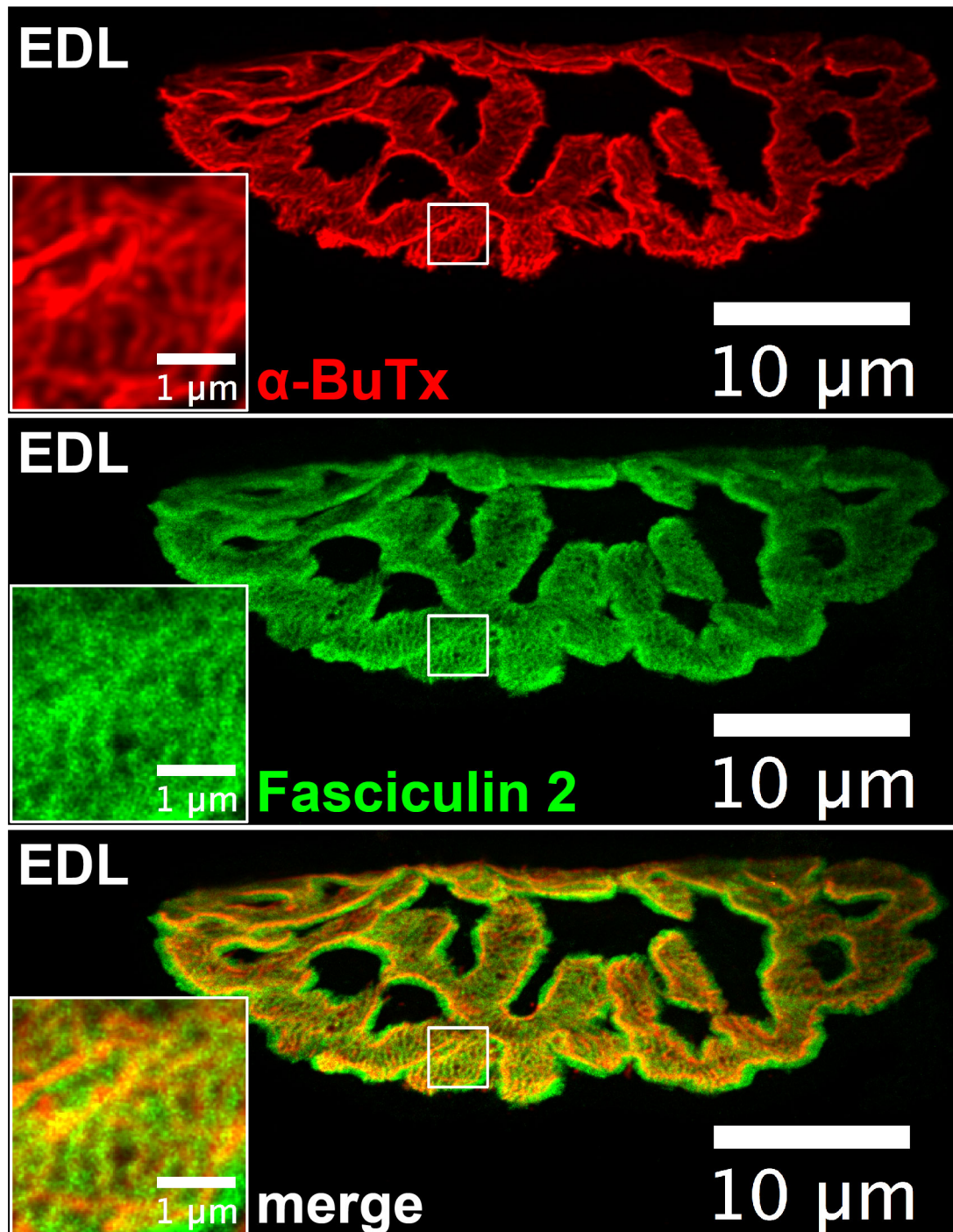


Figure 6-17 Maximum intensity projection gSTED image of an endplate on EDL muscle of $hy^+\epsilon^{het}$ mice

The EDL muscle was dissected from $hy^+\epsilon^{het}$ control mice, and labelled with α -BuTx-594 and fasciculin 2-488. Teased muscle fibres were assessed by confocal microscopy with activated STED592 and STED660 lasers.

In the new era of optical microscopy, the endplate AChR clusters could be studied by stimulated emission depletion (STED) microscopy at higher definition to the confocal stack images (Figures 6-16 and 6-17). However, the achievable improvement in resolution by STED laser is dependent on the working distance of the sample, i.e. NMJ, from the objective, which in the teased muscle fibre preparations will vary. For the same reason, the performance of STED laser within image stacks can be inconsistent. Such challenges remain to be resolved; meanwhile, basic confocal imaging is an option for analysis. (Figures 6-16 and 6-17)

Chapter 7

Conclusions & Future perspectives

7.1 The diagnosis of CMS

The use of next generation sequencing (NGS) will eventually be the preferred method for the detection of CMS-causing mutations in patients. As opposed to Sanger sequencing, which involves testing for mutations in single genes in a targeted and orderly manner, NGS allows the whole genome/exome to be sequenced in unison (Grada and Weinbrecht, 2013). Because the clinical presentation of CMS can be similar to other neuromuscular disorders of infancy, the diagnosis and therapy for a *RAPSN*-CMS patient may be delayed (Das et al., 2014). In addition to improved sensitivity and efficiency in the identification of CMS-causing mutations, the application of NGS has led to the identification of mutations that were previously not known to be associated with CMS (Belaya et al., 2015). Hence it is expected that more CMS causative genes will be acknowledged in the future to allow for better diagnoses and treatment regimens for patients. Despite its advantages, NGS can be an expensive approach, and conducting data analyses of the huge amount of data generated could be a time consuming process. It has been suggested that targeted sequencing of a panel of disease-specific genes is a preferred and more affordable diagnostic approach (Grada and Weinbrecht, 2013; Xuan et al., 2013). As such, information from clinical examination would still determine if targeted sequencing of CMS-associated genes should be performed.

Defining gene variants that cause CMS is crucial for making a final diagnosis. It is often challenging to make a diagnosis for *RAPSN*-CMS that does not involve the common mutation N88K. The functional analysis of novel *RAPSN* variants shows reduction in AChR clustering, and confirmed that the patients who harbour these variants are affected by CMS due to *RAPSN* mutation. Thus the 10 novel missense variants confirmed to cause *RAPSN*-CMS, together with the 20 previously reported missense mutations in *RAPSN*, allows future diagnoses to be made more efficiently.

7.2 Mechanisms of *RAPSN* mutations

Functional analyses of the *RAPSN* mutations V45M, N88K, R91L and A153T reaffirmed that the pathogenic mechanisms of *RAPSN* mutations are diverse (Cossins et al., 2006), but the formation of unstable AChR clusters *in vitro* appears to be a common pathway. Therefore, these mutations are also likely to reduce the stability of postsynaptic endplate structures, and therapies that stabilise the NMJ structure would benefit *RAPSN*-CMS patients.

The mutations may cause an impaired interaction between rapsyn and calpain, since this was found to stabilise AChR clusters by suppressing the calpain-mediated Cdk5 signalling that regulates AChR disassembly upon cholinergic stimulation (Chen et al., 2007). Further studies may reveal ways of stabilising the AChR clusters and thus will provide pointers for potential new treatments. This is important as sudden respiratory crises are life threatening, and occasional deaths from these conditions still occur. However, there are gaps in the knowledge of the agrin-induced MuSK signalling cascade for the formation and maintenance of AChR clusters.

Studies have identified that agrin stimulates the phosphorylation of Dok-7 and leads to the recruitment of adapter proteins Crk and Crk-L (Hallock et al., 2010). The inactivation of Crk and Crk-L resulted in severe NMJ defects, demonstrating that Dok-7 functions downstream from MuSK (Hallock et al., 2010). The subsequent signalling events that direct or regulate rapsyn to cluster AChR are yet to be identified. Improved understanding of the signalling pathways that regulates AChR clustering could allow for further investigations into the mechanisms of *RAPSN* mutations in causing AChR deficiency as well as the identification of new candidate genes that may cause CMS.

Reduced protein stability of rapsyn-EGFP is another common functional effect of the mutations studied. The molecular chaperone, heat shock protein 90 β (HSP90 β), also interacts with rapsyn, and was found to be necessary for both rapsyn stabilisation and the maintenance of AChR clusters *in vivo* (Luo et al., 2008). Interestingly, the TPR domain, in which the investigated mutations reside, was found to be necessary for rapsyn to interact with HSP90 β or calpain (Chen et al., 2007; Luo et al., 2008).

To further dissect the mechanisms of AChR deficiency due to *RAPSN* mutations, other interacting partners of rapsyn that have been suggested to regulate AChR clustering (summarised in Table 7-1) could be investigated in the future.

Table 7-1 Interacting partners of rapsyn that *RAPSN* mutations may impact on

Interacting partners of rapsyn	Domain of rapsyn	Dependent on agrin	Proposed function and regulation	References
α -actinin	Coiled-coil*	Yes	Formation and maintenance of AChR clusters, anchor AChR clusters to actin cytoskeleton	(Dobbins et al., 2008)
Actin-binding domain of ACF7	TPR	Not known	Anchor AChR clusters to actin cytoskeleton	(Antolik et al., 2007)
β -catenin	Not known	Yes	Modulates agrin-induced AChR clustering; and proposed to anchor AChR clusters to filament-actin cytoskeleton via α -catenin	(Zhang et al., 2007)
β -dystroglycan	Not known	Not known	Anchor AChR clusters to the actin cytoskeleton via dystroglycan complex	(Cartaud et al., 1998)
Calpain	TPR	Yes	Calpain activates Cdk5 signalling for AChR cluster disassembly, but its interaction with rapsyn stabilises AChR clusters	(Chen et al., 2007)
CK2	TPR6	Not known	Unknown, but CK2 phosphorylates MuSK to promote the maintenance of AChR clusters	(Cheusova et al., 2006; Herrmann et al., 2015)
HSP90 β	TPR	Yes	Stabilise rapsyn expression, formation and maintenance of AChR clusters	(Luo et al., 2008)
Plectin 1f	Not known	Yes	Formation and maintenance of AChR clusters; and bridge AChR clusters to the post-synaptic intermediate filament network	(Mihailovska et al., 2014)
PKA type 1	Coiled-coil	Not known	Stabilise AChR expression and maintenance of AChR clusters	(Choi et al., 2012a)
Coiled-coil*, the domain is required but insufficient for the interaction between rapsyn and α -actinin				

7.3 Mechanisms of the *Chrnd* mutation, E381K, that mimics *RAPSN*-CMS

Another crucial piece of information for understanding the pathogenic mechanisms of *RAPSN* mutations is the precise interactions between rapsyn and AChR. The loss of stability of the AChR clusters caused by the *Chrnd* mutation E381K showed similarities to the analysis of *RAPSN* mutations. The patient who harboured the *CHRND* mutation E381K was reported to have had recurrent respiratory crises (Muller et al., 2006b), which is also a common feature of *RAPSN*-CMS patients. The findings suggest the δ -subunit of AChR is involved in the clustering of the receptors with rapsyn.

It is well known that tyrosine phosphorylation of the β -subunit of the AChR is involved in regulating the binding of rapsyn to the receptors (Borges et al., 2008). Although agrin also induces tyrosine phosphorylation of the δ -subunit (Colledge and Froehner, 1997), the involvement of δ -subunit in the interaction between rapsyn and AChR was believed to be dispensable (Lee et al., 2009). It has been proposed that the antagonistic effects of the serine phosphorylation of AChR subunits by PKA (δ - and γ -/ ϵ - subunits) and PKC (α - and δ - subunits) could act to regulate the trafficking and metabolic stability of AChR (Lanuza et al., 2006; Martinez-Pena y Valenzuela et al., 2013). PKC induced phosphorylation of δ -subunit at δ^{Ser378} , δ^{Ser393} and δ^{Ser450} has been found to result in the dispersal of AChR clusters (Nimnual et al., 1998). Studies on the dynamics of AChR and rapsyn in clusters have found that PKC activation reduced the insertion rate of AChR into clusters, but not rapsyn, and that PKA activation had no effects (Bruneau and Akaaboune, 2007).

The finding that the *Chrnd* mutation E381K, which is in close proximity to the serine residues, reduced the stability of AChR clusters supports the hypothesis that the δ -subunit could be involved in regulating the cell surface distribution of the receptors through PKC activities (Nimnual et al., 1998). It also highlights a probable role for rapsyn in the maintenance of AChR clusters by mediating the phosphorylation states of the receptors. Rapsyn is thought to anchor PKA to sites in AChR clusters, which may counteract the activities of PKC phosphorylation and stabilise AChR clusters (Choi et al., 2012a; Martinez-Pena y Valenzuela et al., 2013). In addition, rapsyn is thought to interact with casein kinase 2, which phosphorylates serine residues on MuSK and also Dok-7 to promote the stability of AChR clusters (Cheusova et al., 2006; Herrmann et al., 2015). Further investigations into the consequences of *RAPSN* mutations and E381K in *CHRND* on the phosphorylation states of the AChR subunits may provide an insight into the role of phosphorylation in the stability of AChR clusters and how mutations impair the process.

7.4 Application of CRISPR/Cas9 in CMS research

CRISPR/Cas9 mediated genome engineering is a major scientific advancement that could benefit biomedicine from basic research to human gene therapy (Yang, 2015). This thesis shows the first steps of using CRISPR-Cas9n as a tool for analysing mutations identified from postsynaptic-CMS in cultured muscle cells.

While the targeted mutagenesis by CRISPR-Cas9n might be the desirable option for investigating the pathogenic effects of endogenous protein variants, the technical

aspects proved to be problematic. The difficulties in the generation of mutant cell lines and the clonal heterogeneity in C2C12 cells could stem from it being a polyploid cell line. The use of primary muscle cells that are not polyploids may allow reliable investigations into the effects of mutations to be performed. Alternatively, induced pluripotent stem cells derived from patients may offer an alternative solution, as these cells carry the exact genetic background of patients for disease modelling (Salani et al., 2012; Shoji et al., 2015).

On the other hand, gene knockout of *Chrnd* induced by CRISPR-Cas9n and the overexpression of *Chrnd* variants in C2C12 allows more control over variables that could affect the experimental results. The cell line KO6 is currently to be used by colleagues in our laboratory to investigate the effect of a novel variant of *CHRND* identified from a CMS patient. This suggests that gene knockout for other postsynaptic CMS-associated genes could be generated relatively easily with CRISPR-Cas9n, and these cells could be integrated into functional analysis systems to understand the pathogenic properties of mutations causing CMS phenotypes.

Gene editing via the CRISPR/Cas9 system to restore dystrophin expression is currently being explored as a therapeutic strategy for Duchenne muscular dystrophy (Ousterout et al., 2015). Since CMS is comprised of a group of disorders that stem from genetic mutations, the application of CRISPR/Cas9 to correct gene mutations could also provide an alternative therapeutic approach. However, the methods of delivery and the specificity of the target effects of Cas9 activities require further investigations.

7.5 Salbutamol as an additional therapeutic regimen for AChR deficiency syndromes

For AChR deficiency patients, pyridostigmine is currently used as the first-line treatment to compensate for the reduced sensitivity in neuromuscular transmission (Engel et al., 2015). The finding that suggested prolonged pyridostigmine treatments cause disruptions to the endplate AChR clusters on the soleus muscle agrees with research that showed different muscles can have variable sensitivities to pyridostigmine treatments (Lintern et al., 1997). Prolonged activation of AChR can lead to the desensitisation of the receptors and block neuromuscular transmission (Franke et al., 1992). The complete inactivation of acetylcholinesterase increased the removal rate of AChR at the NMJ (Krejci et al., 2006), which suggests that chronic pyridostigmine therapy may destabilise clustered AChR at the NMJ.

The mechanism of salbutamol action is still to be determined. Salbutamol may increase cAMP signalling and in turn regulates PKA activities, which may act to stabilise AChR at endplates. Supporting this hypothesis, salbutamol provides beneficial effects for *COLQ* and *DOK7*-CMS patients (Liewluck et al., 2011). These 2 types of CMS are generally perceived to be associated with unstable postsynaptic structures. Dok7 activates MuSK (Bergamin et al., 2010), and ColQ tethers acetylcholinesterase to MuSK on the postsynaptic membrane (Karmouch et al., 2013).

Elevated cAMP signalling extends the half-lives of AChR on denervated muscles and cultured myotubes (O'Malley et al., 1993; Shyng et al., 1991). PKA is implicated in the turnover and recycling of AChR, which influences receptor density at the NMJ (Martinez-Pena y Valenzuela et al., 2013). It has been shown that reduced levels of

PKA at subsynaptic regions reduce the size of AChR clusters and induces fragmentation (Roder et al., 2010). By contrast, cAMP signalling, by the action of PKA, prevents fragmentation and slows AChR degradation on denervated muscles (Roder et al., 2010; Roder et al., 2012).

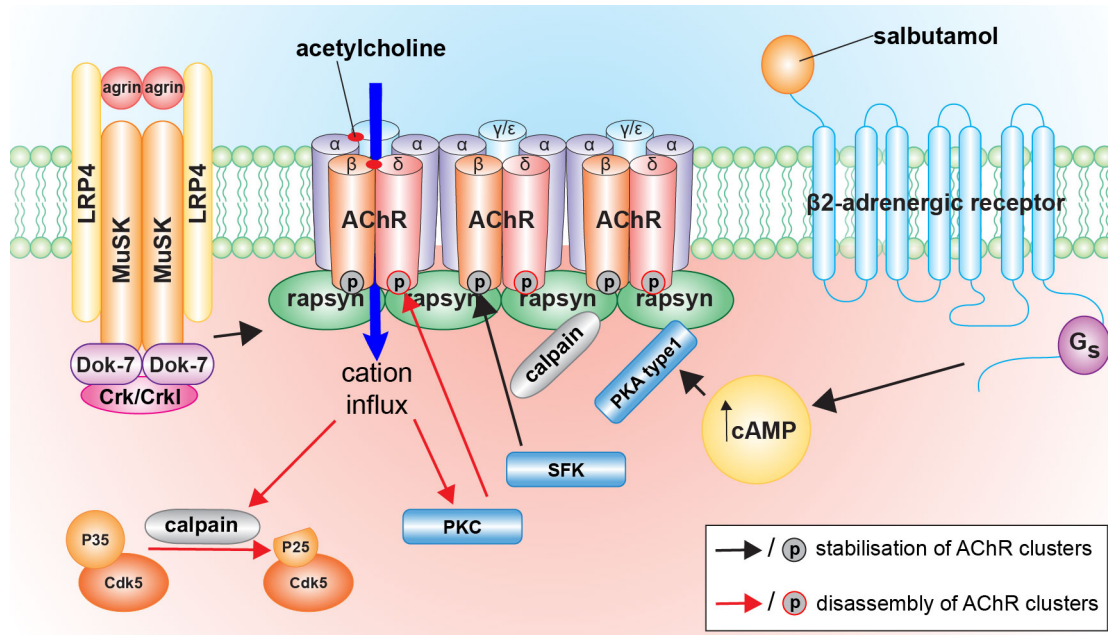


Figure 7-1 Signalling pathways that regulates the stability of AChR clusters

The agrin-induced MuSK signaling, phosphorylation of the β -subunit of AChR by Src-family kinases (SFK) as well as the activities of cAMP-dependent protein kinase A (PKA) has been reported to stabilise AChR clusters.

Cholinergic stimulation of AChR leads to cation influx, which has been reported to induce calpain-mediated activation of Cdk5 and subsequent disassembly of AChR clusters. It has been reported that rapsyn interacts with calpain to prevent the cleavage of p35 to p25, which regulates the activities of Cdk5. The influx of cations can also activate the calcium ion dependent protein kinase C (PKC), which phosphorylates the δ -subunit of the AChR. This process has also been reported to be associated with the dispersion of AChR clusters.

Signalling pathways that regulate the stability of AChR clusters are complex and not fully understood. It has been shown that the opposing effects of PKA and PKC regulate the dynamics of AChR *in vivo*. The precise mode of action of salbutamol at the NMJ is yet to be determined, but it is possible for salbutamol to up-regulate cAMP levels and enhance PKA activities.

Rapsyn is thought to be involved in the anchoring of PKA at the subsynaptic region for stability (Choi et al., 2012a). It is therefore possible for salbutamol to provide additional PKA activities, to compensate for defective rapsyn functioning due to *RAPSN* mutations, for the stabilisation of AChR clusters that are otherwise unstable. In *RAPSN*-CMS patients, the greatest cause for concern is the unpredictable life-threatening crises although they are usually resolved with age. If salbutamol was to provide a positive therapeutic effect for *RAPSN*-CMS patients, its use during early childhood might improve the prognosis for these patients.

Salbutamol is also well known to have anabolic effects on skeletal muscles (Carter and Lynch, 1994; Martineau et al., 1992), which stimulate muscle growth and contribute towards increased muscle strength. Intraperitoneal injection of salbutamol at 0.6mg/kg/day induced hypertrophy of all muscle fibre types in the rat soleus muscle and altered fibre type distribution (Soic-Vranic et al., 2005). Similarly, although it did not reach statistical significance, salbutamol treatment caused a trend of increasing the cross sectional area of TA muscles in the mouse model of anti-MuSK myasthenia gravis (Ghazanfari et al., 2014). An analysis of the effects of salbutamol treatment on the size of muscle fibres in $hy^{+/-}\epsilon^{-/-}$ mice may provide an insight into its mode of action. The diaphragm, EDL and TA muscles in mice are mostly composed of fast-twitch fibres, and the soleus muscles have a similar amount of both fast- and slow-twitch fibres (Bloemberg and Quadrilatero, 2012; Guido et al., 2010). It could also be interesting to analyse AChR clusters on muscles based on specific fibre types, as it has been suggested that mutations in CMS may affect fast- and slow-twitch fibres in skeletal muscles differently (Park et al., 2014).

Of note, treatment with salbutamol alone has not been reported to benefit AChR deficiency syndromes. β_2 adrenergic receptors can also be desensitised by prolonged activation (Lohse et al., 1996). Therefore we do not yet know if the benefits will be maintained throughout the life of a patient. Hence the hypothesis that salbutamol could support the accumulation of AChR to a density that counteracts reduced AChR expression to improve neuromuscular transmission warrants investigation.

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