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Diverse molecular mechanisms involved in AChR deficiency due to rapsyn mutations

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Congenital myasthenic syndromes are inherited disorders of neuromuscular transmission characterized by fatigable muscle weakness. Autosomal recessive acetylcholine receptor (AChR) deficiency syndromes, in which levels of this receptor at the neuromuscular junction are severely reduced, may be caused by mutations within genes encoding the AChR or the AChR-clustering protein, rapsyn. Most patients have mutations within the rapsyn coding region and are either homozygous for N88K or heteroallelic for N88K and a second mutation. In some cases the second allele carries a null mutation but in many the mutations are missense, and are located in different functional domains. Little is known about the functional effects of these mutations, but we hypothesize that they would have an effect on AChR clustering by a variety of mechanisms that might correlate with disease severity. Here we expressed *RAPSN* mutations A25V, N88K, R91L, L361R and K373del in TE671 cells and in rapsyn−/− myotubes to determine their pathogenic mechanisms. The A25Vmutation impaired colocalization of rapsyn with AChR and prevented agrin-induced AChR clusters in rapsyn−/− myotubes. In TE671 cells, R91L reduced the ability of rapsyn to self-associate, and K373del-rapsyn was significantly less stable than wild-type. The effects of mutations L361R and N88K were more subtle: in TE671 cells, in comparison with wild-type rapsyn, L361R-rapsyn showed reduced expression/stability, and both N88K-rapsyn and L361R-rapsyn showed significantly reduced co-localization with AChR. N88K-rapsyn and L361R-rapsyn could effectively mediate agrin-induced AChR clusters, but these were reduced in number and were less stable than with wild-type rapsyn. The disease severity of patients harbouring the compound allelic mutations was greater than that of patients with homozygous rapsyn mutation N88K, suggesting that the second mutant allele may largely determine severity.

Keywords: acetylcholine receptor; congenital myasthenic syndrome; neuromuscular junction; rapsyn

Abbreviations: AChR = acetylcholine receptor; α-BuTx = α-bungarotoxin; DMEM = Dulbecco-modified essential medium; MG-ADL = myasthenia gravis activities of daily living; PBS = phosphate-buffered saline; PS = penicillin G and streptomycin; TPR = tetratricopeptide repeat

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Introduction

Synaptic transmission plays a pivotal role in controlling the passage of information through the nervous system. Synaptogenesis involves the juxtaposition of pre- and post-synaptic structures and the generation of regions of densely packed neurotransmitter receptors. At the neuromuscular junction (NMJ) a core pathway involving agrin, muscle-specific tyrosine kinase (MuSK), acetylcholine receptors (AChRs) and the AChR-clustering protein rapsyn is thought to be responsible for maintaining synaptic structure and for the aggregation and localization of AChR on the post-synaptic folds (Sanes and Lichtman, 2001). Although mutations underlying congenital myasthenic syndromes (Engel and Sine, 2005) have been identified in post-synaptically expressed genes encoding MuSK (Chevessier *et al.*, 2004)

and the voltage-gated sodium channel, $Na_v1.4$ (Tsujino *et al.*, 2003), the majority are located in the *AChR* or *rapsyn* genes [a list of these mutations is given in Ohno and Engel (2004)]. AChR mutations have been well studied, but the functional effects of rapsyn genes have not yet been characterized in detail.

In early developmental stages the AChR is expressed throughout muscle, with clusters or 'hot-spots' of AChR forming independently of the nerve in the process of muscle pre-patterning (Kummer *et al.*, 2005). Motoneuron axons then extend into the muscle and release agrin, which activates MuSK through an as-yet undetermined mechanism. AChRs are phosphorylated and their expression becomes restricted to the synaptic and perisynaptic regions of the muscle. As the junction matures, dense, stable clusters of AChR become localized at the crests of the post-synaptic folds of the NMJ. Rapsyn interacts directly with AChRs and is essential for agrin-induced clustering of the AChR (Gautam *et al.*, 1995; Fuhrer *et al.*, 1999). In rapsyn−/− mice AChR clusters fail to form, the post-synaptic folds do not form properly, nerve sprouting occurs and the mice die at birth without functional synapses (Gautam *et al.*, 1995, 1999). AChRs, in turn, are required for synaptic clustering of rapsyn, revealing an intimate functional interdependence of AChRs and rapsyn (Marangi *et al.*, 2001; Ono *et al.*, 2004).

The effect of deletions and truncations of rapsyn on its function *in vitro* have led to the proposal of four functional domains: rapsyn is attached to the juxtaneural post-synaptic membrane by a myristylation moiety; seven tetratricopeptide repeat (TPR) domains are thought to mediate rapsyn self-association (Ramarao *et al.*, 2001; Eckler *et al.*, 2005); a zinc finger/coiled-coil domain has been implicated in interaction of rapsyn with the AChR (Bezakova and Bloch, 1998; Ramarao *et al.*, 2001); and a RING-H2 domain is thought to be involved in rapsyn binding to scaffolding proteins such as dystroglycan (Bartoli *et al.*, 2001).

Mutations within the *RAPSN* gene have been shown to underlie a high proportion of AChR deficiency syndromes (Ohno *et al.*, 2002; Burke *et al.*, 2003; Dunne and Maselli, 2003; Ohno *et al.*, 2003; Maselli *et al.*, 2003; Muller *et al.*, 2003; Ioos *et al.*, 2004), which can occur early in life or during adulthood. The disease is recessive, and in most cases patients are either homozygous for N88K or heteroallelic for N88K and a second mutation. The severity of disease is variable, ranging from little functional impairment to the patient requiring assisted ventilation. In particular, the disorder in childhood is frequently characterized by severe apnoeic attacks. To date the precise mechanisms by which these different *RAPSN* mutations lead to AChR deficiency have not been thoroughly studied.

Here we investigated the functional effects of five *RAPSN* gene mutations, including N88K, and asked whether these correlated with the clinical phenotype. Our data show that diverse molecular mechanisms disrupt rapsyn function, and that the major effect of N88K-rapsyn is to reduce the stability of AChR clusters. However, in compound heterozygotes of N88K and a second mutation, the second mutant allele is likely to play a role in determining disease severity.

Material and methods

Patients with AChR deficiency and the identification of *RAPSN* mutations

Patients and DNA samples were recruited through the congenital myasthenia service in Oxford, UK. All were negative for antibodies against the AChR. Information was from family interviews, case note review and clinical examination obtained by an assessor blinded to the results of the genetic functional analysis. Assessment was complemented by the myasthenia gravis activities of daily living (MG-ADL) scoring system, an eight-question survey of symptom severity, with each response graded from 0 (normal) to 3 (most severe) (Wolfe *et al.*, 1999). Mutations were identified by direct sequencing of polymerase chain reaction (PCR) amplicons containing the eight *RAPSN* exons and their flanking non-coding regions and confirmed by restriction endonuclease digestion as described previously (Burke *et al.*, 2004). Approval for this study was obtained from the Central Oxford Research Ethics Committee.

Plasmids and constructs

Retroviral vector pBabe-PURO-sn was a kind gift from Dr Chris Norbury, Cancer Research UK, WIMM, Oxford. It is adapted from pBabe-PURO (Morgenstern and Land, 1990), such that the multiple cloning site contains *Xho* I, *Sal* I, *Not* I and *Bam*H I restriction sites. pEGFP-N1 was purchased from Clontech. Restriction enzymes were purchased from New England Biolabs. Human rapsyn cDNA minus the stop codon was amplified using primers 5′-TGCGAAGCTTGCCACCATGGGGCAGGACCAGACCAAG and 5′-TCGTGGATCCCGTACAAAGCCAGGCTTCATGGA and was cloned into pEGFP-N1 using *Hin*d III and *Bam*H I restriction sites, such that EGFP and rapsyn were in the same reading frame. Mutagenesis was carried out using the QuikChange mutagenesis kit purchased from Stratagene. Mutations were confirmed by DNA sequencing. EGFP-tagged rapsyn and mutants were excised from pEGFP-N1 using *Hin*d III (filled) and *Not* I, and were cloned into *Xho* I (filled) and *Not* I digested pBabe-PURO-sn.

Cell lines

TE671, a rhabdomyosarcoma with muscle-like properties (Stratton *et al.*, 1989), COS-7 and C2C12 cells were purchased from ATCC and GP + E86 (Markowitz *et al.*, 1988) retroviral-producing cell line was a kind gift from Professor Roy Bicknell, Cancer Research UK, WIMM, Oxford. TE671, COS-7 and GP + E86 cell lines were maintained at 37°C in Dulbecco-modified essential medium (DMEM) (Sigma–Aldrich) supplemented with 10% FCS (TCS Cellworks Ltd) and 100 U/ml each of penicillin G and streptomycin (PS) purchased from Invitrogen. C2C12 cells were maintained at 37°C in DMEM supplemented with 20% FCS and PS, and were fused in DMEM containing 2% FCS and PS. Rapsyn−/− (clone 11–7) myoblasts (Fuhrer *et al.*, 1999) were maintained at 33°C with 5% CO_2 on plates coated with matrigel matrix (BD Biosciences Discovery Labware, San Jose, CA, USA). Growth medium was DMEM supplemented with 0.5% chick embryo extract (United States Biological, MA, USA), 4 U/ml mouse recombinant interferon-γ (Sigma–Aldrich), 20% FCS and PS. To induce fusion, myoblasts were moved to 37°C with 5% CO_2, and the medium was replaced

with fusion medium [DMEM containing 10% horse serum (Sigma–Aldrich) and PS]. Fusion medium was replaced every day until myotubes formed.

Transfections

TE671 cells were seeded at 2×10^5 cells per well in 6-well plates and the following day were transfected with a total of 3 μg DNA per well using calcium phosphate precipitation. Amounts of DNA used per well were 1 μg AChR α-subunit DNA, 0.5 μg each of AChR β-, δ- and ε-subunit cDNA and 0.5 μg of rapsyn cDNA or pcDNA3.1-hygro for 'no rapsyn' control transfections. Transfections with only rapsyn and no AChR were carried out using 3 μg/well of rapsyn expression plasmids. GP + E86 cells were transfected with the pBabe-PURO-rapsyn constructs using Fugene (Roche Diagnostics), using 2.5 μg DNA and 7.5 μl Fugene in a 25 cm^2 flask. Three days following transfection cells were selected using 8 μg/ml puromycin (Sigma–Aldrich), and 2 weeks later were cell-sorted to obtain the top 5% fluorescent cells.

Retroviral production and infection

For retroviral production transfected GP + E86 cells were incubated for 3 days in the minimal volume of growth medium without puromycin. The medium was harvested and centrifuged to remove cell debris, aliquoted and snap frozen on dry ice. Retrovirus was stored at −80°C. Retrovirus was used neat and was incubated with myoblasts overnight at 33°C after which the medium was replaced with growth medium. Puromycin was added 3 days after infection.

Western blots

TE671 cells were removed from 6-well plates using trypsin/EDTA in phosphate-buffered saline (PBS) and centrifuged, and the pellets were resuspended in 5× protein loading buffer. The cell extract was incubated at 95°C for 1 h and then subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) using Novex precast 4–20% NuPage gels. Protein was transferred to nitrocellulose and rapsyn was detected using anti-rapsyn mAb 1234 (Abcam) followed by anti-mouse-HRP and ECL (Amersham). α-Tubulin was detected on the same western blots using an mAb (Sigma–Aldrich) followed by anti-mouse-HRP and ECL.

Production of agrin and generation of AChR clusters on myotubes

To generate soluble rat agrin, COS cells were transiently transfected with construct s-agrin-(4,19), kindly given by Werner Hoch (Hopf and Hoch, 1997), using PEI. On the following day the medium was replaced with fresh medium. Conditioned medium was harvested 2 days later, aliquoted and snap frozen. AChR clustering activity of the agrin was titrated by incubation overnight with C2C12 myotubes. Clusters were labelled at 37°C for 1 h with tetramethylrhodamine α-bungarotoxin (α-BuTx) (Molecular Probes) diluted 1 : 1000 in fusion medium and washed three times with fusion medium. Cells were fixed with 3% paraformaldehyde and cluster number and length were counted using an Axion 200 inverted Zeiss fluorescence microscope. AChR clustering on rapsyn−/− myotubes was carried out by incubation overnight at 37°C with the optimal dilution of agrin in fusion medium. Labelling of clusters was as described for C2C12 cells.

Fluorescence/confocal microscopy

Microscopy was performed on an Axion 200 inverted Zeiss fluorescence microscope, an Olympus BX60 wide-field fluorescence microscope or on a Bio-Rad Radiance 2000 confocal microscope. Some intracellular aggregates of EGFP-tagged rapsyn were observed in a sub-population of cells transfected with all rapsyn-EGFP constructs, including wild-type rapsyn-EGFP. AChR on TE671 cells was detected by incubation for 1 h at room temperature with the B3 mAb, which binds the extracellular domain of the AChR β-subunit (Jacobson *et al.*, 1999), 1 : 500 in DMEM containing 20 mM HEPES and 1% bovine serum albumin (BSA), before being washed three times with PBS. Cells were fixed with 3% paraformaldeyde at room temperature for 20 min, washed three times with PBS and incubated with secondary antibody Alexa Fluor® 594 goat anti-mouse IgG (H+L) (Molecular Probes) diluted 1 : 1000 in PBS containing 1% BSA. Cells were washed 3× in PBS and mounted in fluorescence mounting medium (Dako Cytomation). AChR on myotubes were labelled at 37°C with tetramethylrhodamine α-BuTx (Molecular Probes) diluted 1 : 1000 in fusion medium. Cells were washed 3× in fusion medium, fixed in fusion medium containing 3% formaldehyde for 20 min at room temperature, washed 1× in PBS and mounted in fluorescence mounting medium. Images were captured by using Openlab software (Improvision) or LaserSharp 2000 software (Bio-Rad).

Analysis of colocalization and clustering

Sixteen-bit depth images from a radiance 2000 confocal microscope were analysed blinded using MetaMorph, Universal Imaging Corporation. For the analysis, background noise was reduced. After consultation with Universal Imaging Corporation the following method was chosen because it gave the best reduction in background with minimal change to the fluorescence of the cell. First a morphological H-Dome, threshold set to 36 000 grey levels, was run. A binarize operation was performed with manual thresholding, and then single pixels were removed. A logical AND was performed between the resulting image and the original image, so that maximum intensity values were identical to those in the original image. Individual cells were selected as regions of interest and all pixels therein were selected by thresholding, including any large aggregates within the cell. Colocalization of rapsyn with AChR was quantified as per cent of green pixels colocalizing with red pixels, and, conversely, colocalization of AChR with rapsyn was quantified as per cent of red pixels colocalizing with green pixels. Densitometry of rapsyn and AChR and area of clusters was also analysed using MetaMorph.

Results

Patients

Eighteen different mutations within or affecting the *RAPSN* coding region were identified in patients referred to the congenital myasthenia service in Oxford, UK, with CMS associated with AChR deficiency (Fig. 1). We chose five of these mutations, A25V, N88K, R91L, L361R and K373del, for functional studies to determine the underlying basis for the end-plate AChR deficiency. These included four missense mutations and one single amino acid deletion within rapsyn. The patients with these mutations are described in Table 1 and include two pairs of siblings, Patients 4 and 5, and 12 and 14. The age at onset varied from birth (10 patients) to later

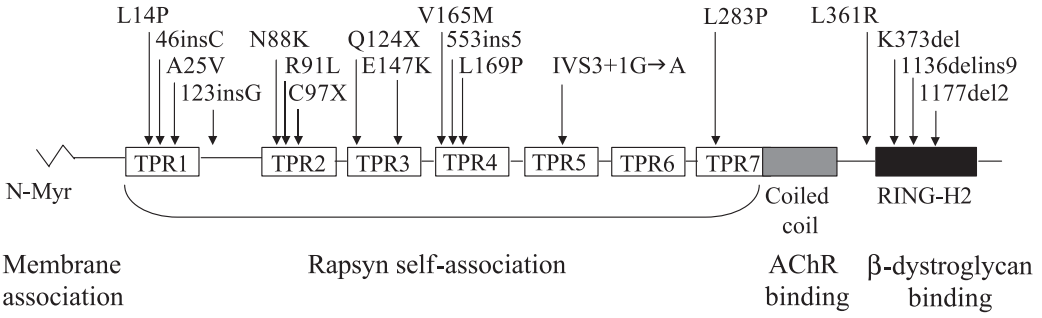

Fig. 1 Schematic diagram of rapsyn protein showing mutations that have been identified in congenital myasthenic patients referred to the Congenital Myasthenia Service in Oxford, UK. The functional domains of rapsyn are also shown.

Table 1 Patient data

Patient no.	Age of onset/sex/ current age (years)	AMC	Assisted ventilation	Apnoeas	Treatment	MG-ADL (/24)	Mutation
1	Birth/M/55	–	+	–	P	ND	A25V, N88K
2	10/M/17	–	–	–	P	7	R91L, N88K
3	Birth/F/1	+	+	+	N	10	K373del, N88K
4	Birth/M/11	–	–	+	P+D	5	K373del, N88K
5	Birth/M/13	–	+	+	P+D	5	K373del, N88K
6	Birth/F/10	+	+	+	P	4	L361R, N88K
7	Birth/F/4	+	+	–	P	4	N88K, N88K
8	Birth/M/7	+	–	+	P	2	N88K, N88K
9	Birth/F/23	+	+	–	P	1	N88K, N88K
10	Birth/M/26	+	–	+	P	2	N88K, N88K
11	Birth/M/30	–	–	–	P	6	N88K, N88K
12	26/F/52	–	–	–	P	6	N88K, N88K
13	48/M/53	–	–	–	P	1	N88K, N88K
14	21/M/54	–	–	–	P	0	N88K, N88K
15	41/F/76	–	–	–	P	0	N88K, N88K

M, male; F, female; AMC, arthrogryposis multiplex congenita; MG-ADL, myasthenia gravis activities of daily living score; ND, not done; P, pyridostigmine; N, neostigmine; D, 3,4-diaminopyridine.

childhood (1 patient) or adult life. In affected individuals each of these mutations was found to be heteroallelic with mutation N88K or homozygous for N88K.

Four of nine homozygous for N88K had arthrogryposis multiplex congenita (AMC) but congenital joint contractures were also evident in two other patients with compound heteroallelic mutations. Six of the patients with symptoms from birth had at least one exacerbation during childhood that required hospital admission, often with assisted ventilation during the attacks. Apnoea attacks occurred in four of the six patients with heteroallelic mutations (three patients with the rapsyn mutation K373del, and in Patient 6 with mutations L361R and N88K), but only in 2/9 rapsyn patients homozygous for N88K, although this difference did not reach significance. Patient 1 (rapsyn mutations A25V and N88K) and the siblings with rapsyn mutations K373del and N88K had older brothers with a similar phenotype to them, but who had died in infancy from respiratory failure.

All but Patient 1 has been examined, usually on treatment, within the last 2 years. At his last examination, Patient 1 (rapsyn mutations A25V and N88K) had some mild weakness of his neck and upper limbs and mild bulbar and ocular symptoms. Patient 2 (rapsyn mutations R91L and N88K) continues to have severe weakness despite treatment with pyridostigmine. Patient 3 (rapsyn mutations K373del and N88K) has mild limb weakness but significant bulbar difficulties on neostigmine. She also requires assisted ventilation at night for nocturnal hypoventilation. Patients 4 and 5 have mild weakness on examination but moderate impairment of activities of daily living, and require treatment with both pyridostigmine and 3,4-diaminopyridine. Patient 6 (rapsyn mutations L361R and N88K) and the patients homozygous for N88K have little or no weakness with pyridostigmine treatment, and this is generally reflected in their low MG-ADL scores (Table 1), which differ significantly between those homozygous for N88K and those with compound heteroallelic mutations (Mann–Whitney $P = 0.029$). No clear relationship between the location of the identified mutations and the disease severity was apparent.

Functional analysis of *RAPSN* mutations

To examine whether the clinical phenotype reflected the functional effects of the mutations on AChR clustering *in vitro*,

cDNA encoding human rapsyn (Buckel *et al.*, 1996) was cloned into mammalian expression vector pcDNA3.1 or given a C-terminal EGFP tag using pEGFP-N1. Preliminary experiments showed that inclusion of the C-terminal EGFP tag did not affect rapsyn-induced clustering of the AChR in the TE671 muscle cell line (data not shown). Subsequently, respective mutations A25V, N88K, R91L, L361R and K373del were introduced into the rapsyn-EGFP expression plasmid for the functional studies.

R91L inhibits rapsyn self-clustering

We first studied the effect of the mutations on rapsyn self-association, which is a critical process in the clustering of the AChR. To do this, plasmids constructed to express wild-type or mutant rapsyn-EGFP were transfected into TE671 cells, images were captured on a confocal microscope (Fig. 2A–F) and the per cent of fluorescent cells that contained clusters was counted. Western blots showed that R91L-rapsyn-EGFP shows equivalent expression and stability to wild-type rapsyn-EGFP and that the diffuse cellular EGFP signal was not due to protein degradation or EGFP clipping (data not shown). R91L-rapsyn showed a significant reduction in self-association, with only 48% of fluorescent cells containing clusters compared with 70% for wild-type (Fig. 2G; repeated-measures ANOVA, $P = 0.006$, with post-test for rapsyn R91L, $P < 0.05$). Much of the fluorescent signal was from large intracellular aggregates (Fig. 2D). In contrast, self-clustering in all the other rapsyn constructs was similar to the wild-type. Since rapsyn self-association is crucial for AChR clustering, it is likely that this is the pathogenic mechanism for this mutation, and it was not examined further.

Mutation K373del reduces the stability of rapsyn

We next analysed the expression levels of the remaining four rapsyn mutants by western blotting. Wild-type or mutant rapsyn-EGFP was transfected on Day 0 (with or without the AChR subunits) into TE671 cells, and a time course for levels of rapsyn and α-tubulin (control) was carried out. An example of TE671 cells transfected with rapsyn alone is shown in Fig. 3A, and similar results were obtained when cells were co-transfected with AChR (data not shown). Densitometry was performed to obtain a ratio of rapsyn : α-tubulin and the results were normalized to levels of wild-type rapsyn-EGFP (Fig. 3B). K373del-rapsyn-EGFP expression drastically decreased to ~10% of that of wild-type rapsyn by Day 4 and was undetectable by Day 5. This initial robust expression of K373del followed by rapid loss strongly suggests that deletion of K373 reduces the stability of rapsyn to a much greater extent than the other mutations. The same results were obtained with a second set of DNA preparations. A25V and L361R also reduced rapsyn levels to ~30%, with a more rapid decline than wild-type in expression between Days 4 and 5, suggesting that the stability of these mutants might also be impaired, albeit to a lesser

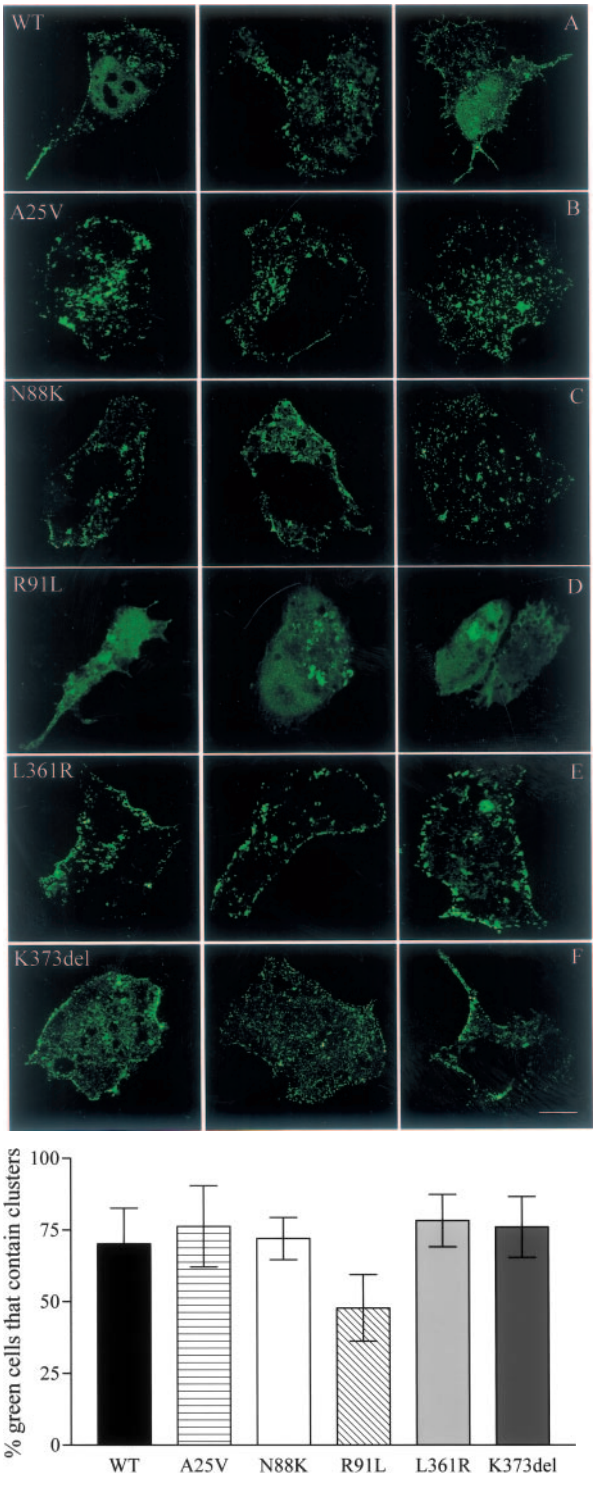

Fig. 2 Mutation R91L abrogated clustering of rapsyn in TE671 cells. (**A**–**F**) Confocal images of wild-type and mutant rapsyn-EGFP in TE671 cells 2 days following transfection, with each column representing a different cell. Rapsyn R91L (**D**) showed reduced self-clustering, but the other mutants (**B, C, E** and **F**) were similar to wild-type (**A**). Magnification: 60×. Bar = 10 μm (**G**) Quantification of rapsyn clustering using wide-field fluorescence microscopy. Number of green fluorescent cells containing rapsyn clusters was expressed as a percentage of total number of green fluorescent cells in 40 fields. Results are the average of four experiments.

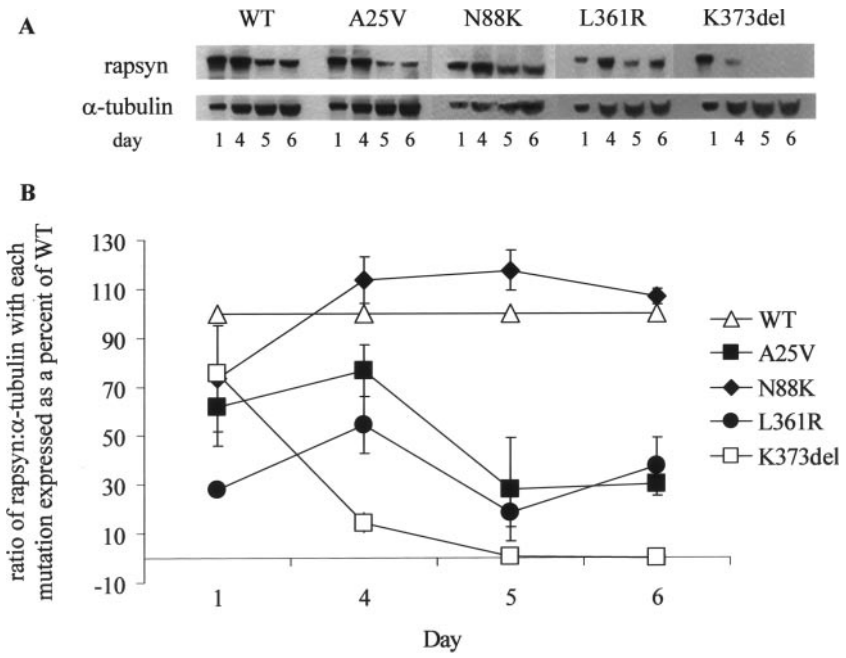

Fig. 3 K373del reduced the stability of rapsyn. (**A**) Wild-type and mutant rapsyn-EGFP were transfected into TE671 cells and analysed by western blotting. Rapsyn was detected by mAb clone 1234 and α-tubulin was labelled as a control. (**B**) The ratio of rapsyn : α-tubulin was obtained by carrying out densitometry using LabWorks. Expression of K373del-rapsyn-EGFP decreased rapidly compared with wild-type rapsyn, indicating that it is unstable. A25V and L361R were also unstable but to a lesser degree, and N88K-rapsyn-EGFP was as stable as wild-type rapsyn (n = 2).

degree than K373del. Interestingly, N88K had no effect on rapsyn expression.

Analysis by flow cytometry of the co-transfected TE671 cells confirmed the results from western blots and also showed that the overall level of cell surface expression of AChR (cells immunolabelled with AChR β-subunit-specific MAb B3) was unaffected by the rapsyn mutations studied here (data not shown). The dramatic effect of K373del on rapsyn stability is likely to be the major molecular mechanism underlying rapsyn dysfunction for this mutation.

A25V-rapsyn does not associate with the AChR and abrogates AChR clustering

Next we investigated the effect of the remaining three mutations, A25V, N88K and L361R, on the interactions between rapsyn and AChR by looking at colocalization of the two proteins. First, in a blinded experiment, TE671 cells were co-transfected with wild-type or mutant rapsyn-EGFP constructs and the AChR cDNAs. Two days following transfection the AChR was immunolabelled and the cells were fixed and mounted. Photographs were taken using a confocal microscope, and colocalization of rapsyn with AChR (per cent of green pixels that are also red) and AChR with rapsyn (per cent of red pixels that are also green) was analysed using MetaMorph. Figure 4A–D show examples of confocal images of the green fluorescent rapsyn constructs, AChR labelled with MAb B3 fluorescing red and merged images. Wild-type rapsyn-EGFP showed the best colocalization with 62% colocalizing with the AChR and 52% of AChR colocalizing with rapsyn-EGFP (Fig. 4E). A25V-rapsyn-EGFP showed the least colocalization, with only 10% of rapsyn colocalizing with the AChR and 17% of AChR colocalizing with rapsyn (Fig. 4E). A25V-rapsyn did not localize at the cell surface; rather A25V-rapsyn-GFP clusters were bigger and appeared intracellularly, in the middle of the cell soma. The AChR signal at the cell surface, in the A25V-rapsyn-EGFP/AChR co-transfections, showed minimal clustering (Fig. 4B) as would be expected in the absence of functional rapsyn. Rapsyn harbouring mutations N88K and L361R showed ~50% reduction in levels of colocalization of both rapsyn with AChR and AChR with rapsyn compared with wild-type in TE671 cells (Fig. 4E).

To further understand the effect of the A25V mutation on co-localization we studied agrin-induced AChR clusters. Wild-type or A25V-rapsyn-EGFP were introduced into mouse rapsyn−/− myoblasts using retroviruses, myoblasts were fused and incubated with agrin overnight and AChR clusters were visualized using α-BuTx-TRITC. Many clusters were induced, as expected, in myotubes expressing wild-type rapsyn-EGFP (Fig. 5A), but clusters in A25V-rapsyn-EGFP myotubes were virtually undetectable (Fig. 5B), whereas they were present with N88K and L361R (Fig. 5C and D). The number of clusters longer than 3 μm was counted in each field. The A25V mutation reduced the number of clusters by 97.5% compared with that of wild-type rapsyn-EGFP (Fig. 5E). As expected, no clusters were observed in agrin-treated rapsyn−/− myotubes (data not shown).

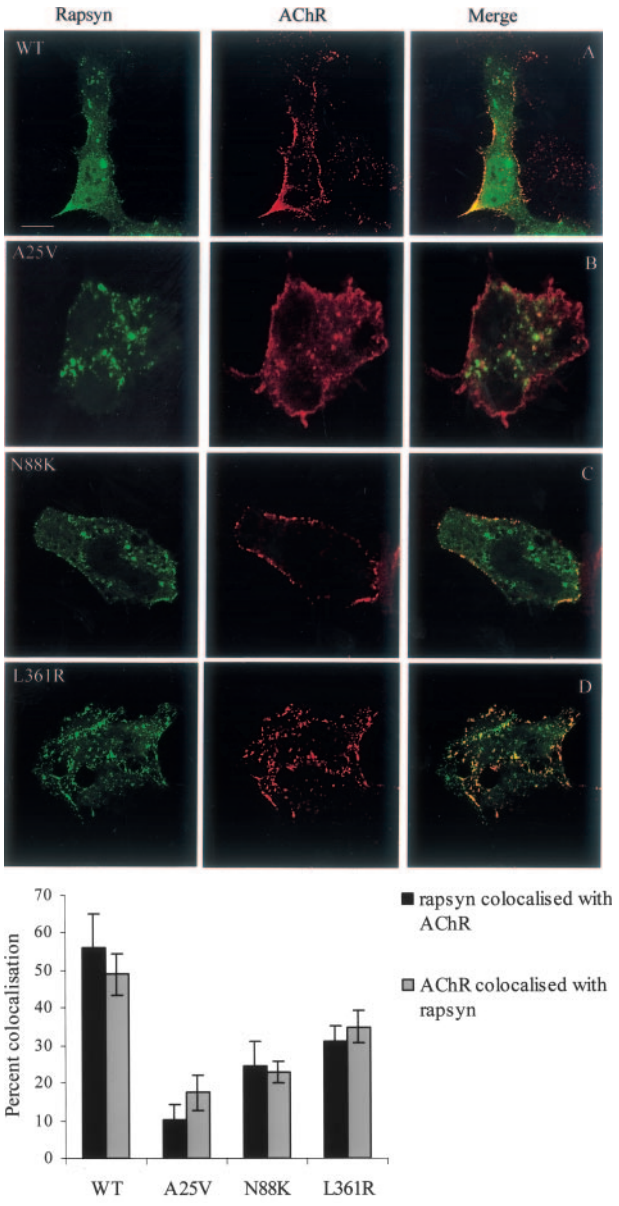

Fig. 4 Mutation A25V reduced colocalization of rapsyn with the AChR. TE671 cells were co-transfected with wild-type, A25V-, N88K- or L361R-rapsyn-EGFP and the AChR subunits. AChR was detected using mAb B3 and images were taken using a confocal microscope. (**A**) Confocal images showing rapsyn (green), AChR (red) and merged images (yellow where rapsyn and AChR colocalize) in TE671 cells, 2 days following transfection. Wild-type, N88K- and L361R-rapsyn-EGFP all showed good colocalization (**A, C** and **D**). Very little colocalization of rapsyn and AChR was observed with A25V-rapsyn-EGFP (**B**) and AChR was only minimally clustered but was evenly distributed on the cell surface. Magnification: 60×. Bar = 10 μm. (**E**) MetaMorph was used to calculate colocalization of rapsyn with AChR (per cent green pixels that are red) and AChR with rapsyn (per cent red pixels that are green).

Confocal images showed that the AChR was located along the cell surface of myotubes. In merged confocal images of agrin-treated myotubes expressing A25V-rapsyn-EGFP, the AChR appeared as red lines along the edge of the myotubes, whereas A25V-rapsyn-EGFP was green inside the myotubes, indicating that rapsyn was not located at the cell surface and did not colocalize with the AChR (Fig. 5G), confirming the results shown in TE671 cells (Fig. 4B). In contrast, in merged images of myotubes expressing wild-type rapsyn-EGFP, in addition to the presence of clusters, the edges of the myotubes were yellow, indicating that rapsyn reached the cell surface and colocalized precisely with unclustered AChR (Fig. 5F).

N88K- and L361R-rapsyn-EGFP form agrin-induced clusters that are unstable

Despite the reduction in colocalization of N88K- and L361R-rapsyn-EGFP with AChR in TE671 cells (Fig. 4E), they were still able to effectively mediate agrin-induced AChR clusters in rapsyn−/− myotubes (Fig. 5C and D). However, the number of AChR clusters was reduced by 30% for N88K and by as much as 60% for L361R (Fig. 5E). The clusters that did form were similar to those formed by wild-type rapsyn-EGFP with respect to average area, density of rapsyn-EGFP, density of AChR and colocalization of rapsyn with AChR (data not shown).

The reduction in number of clusters could be due to less efficient cluster formation or to clusters being unstable once formed. To test this myotubes were incubated overnight with agrin, washed three times to remove the agrin and incubated in differentiation medium for 5 hours before labelling with α-BuTx-TRITC (Fig. 6A–F). Over the 5 hour incubation period the number of AChR clusters per field with wild-type rapsyn-EGFP decreased by only 10% (Fig. 6G), indicating that the clusters are relatively stable. In contrast, with N88K- or L361R-rapsyn-EGFP the AChR clusters were disassembled as reflected by the 85% reduction in the number of clusters per field (Fig. 6G).

Discussion

We studied four missense mutations and one single amino acid deletion within rapsyn that are present in patients with congenital myasthenia owing to end-plate AChR deficiency. They were found to disrupt rapsyn function via different intracellular molecular mechanisms. R91L significantly reduces rapsyn self-clustering, K373del leads to rapid degradation compared with wild-type rapsyn and A25V annuls the association of rapsyn and AChR. The pathogenic mechanisms of N88K and L361R appear to be more subtle. Mutation L361R reduces the level of rapsyn either through lower expression or increased turnover. Both L361R and N88K affect the association of rapsyn with AChR, reduce the number of agrin-induced AChR clusters in rapsyn−/− myotubes and dramatically reduce the stability of AChR clusters. A summary of the results is shown in Table 2.

The functional effects of our mutations do not obviously correlate with the previously proposed functional domains of rapsyn derived from deletion experiments, for instance mutations N88K and R91L are both within TPR2 but have

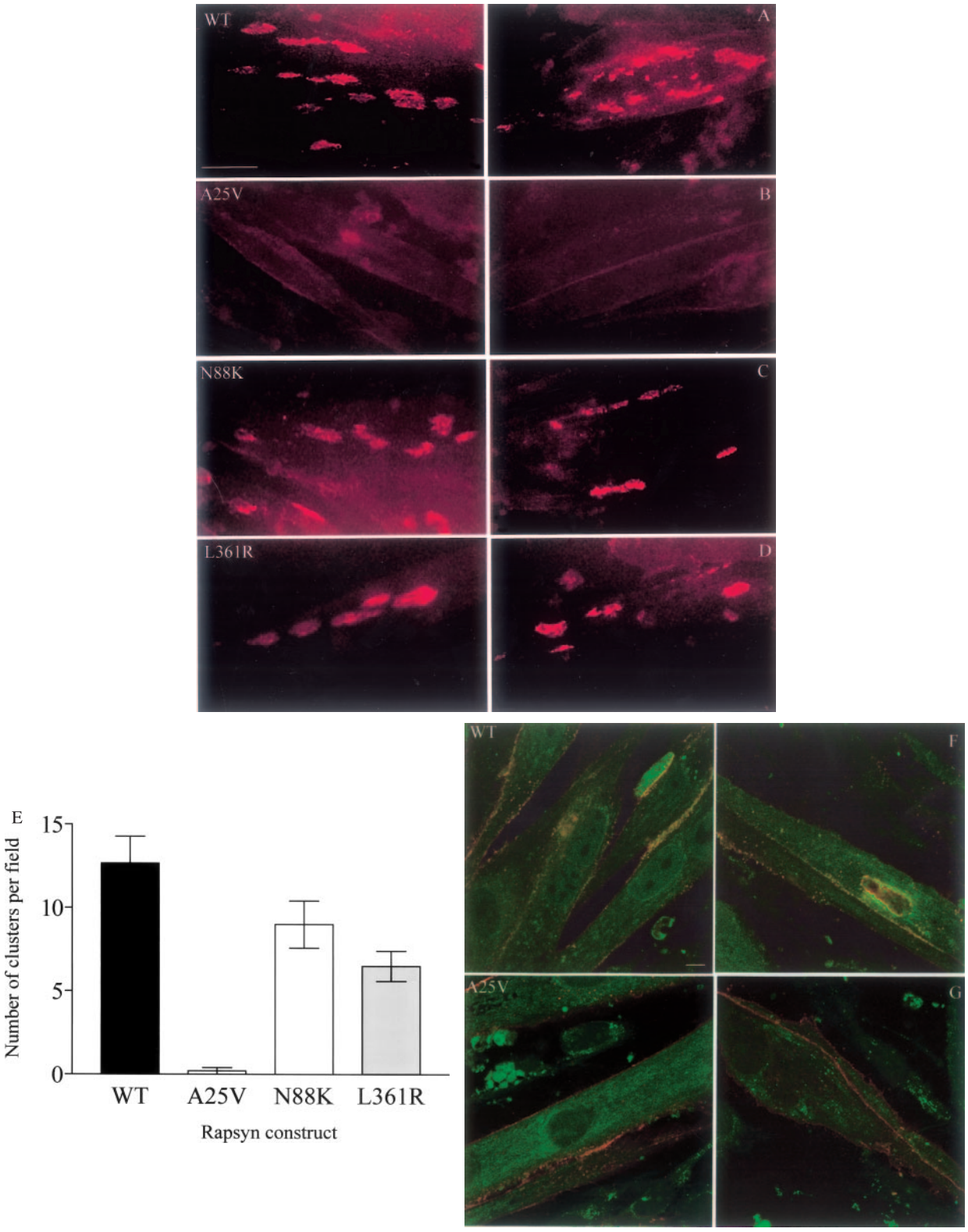

Fig. 5 A25V-rapsyn-EGFP did not form agrin-induced clusters in rapsyn−/− myotubes. Myotubes expressing wild-type, A25V-, N88K- or L361R-rapsyn-EGFP were treated with rat-soluble agrin overnight and AChR was labelled with BuTx-TRITC. Images of 40 fields per construct were taken using a wide-field inverted fluorescence microscope, and example images of AChR clusters are shown (**A**–**D**). Magnification: 32×. Bar = 40 μm. The number of clusters longer than 3 μm were counted and the average number of clusters per field was calculated (**E**). Mutant A25V inhibited the formation of AChR clusters. Confocal images of rapsyn−/− myotubes expressing wild-type rapsyn-EGFP (**F**) or A25V-rapsyn-EGFP (**G**) indicated that A25V-rapsyn-EGFP did not reach the cell surface. Magnification: 60×. Bar = 10 μm.

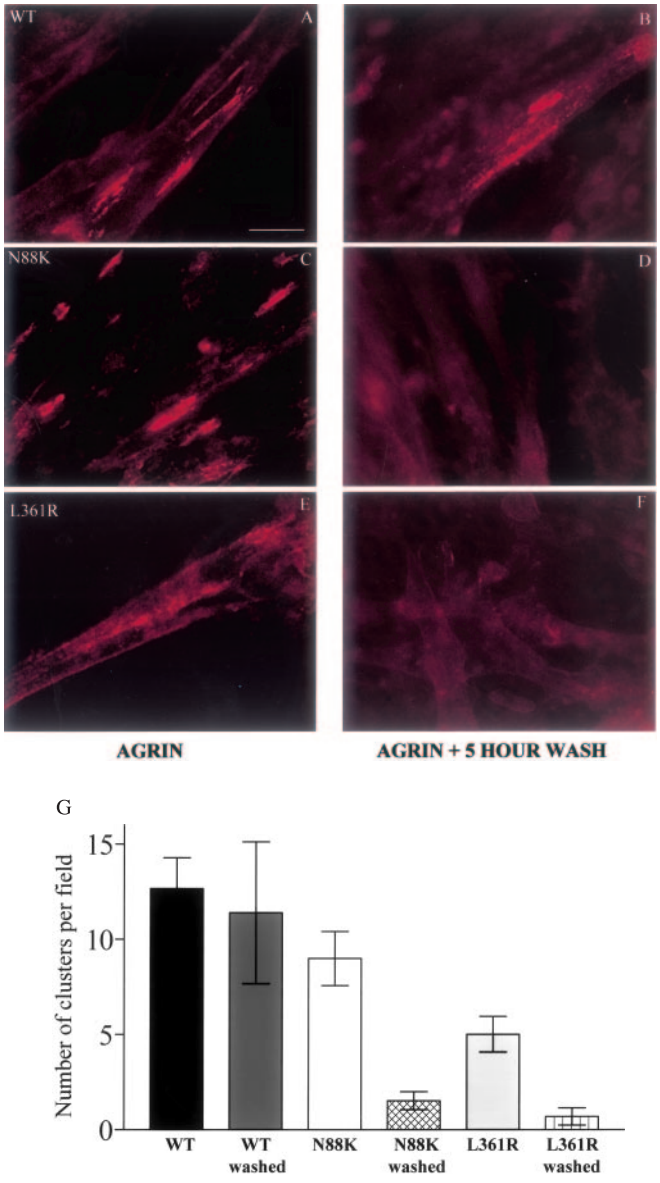

Fig. 6 Agrin-induced AChR clusters that formed with N88K- or L361R-rapsyn-EGFP were unstable. Rapsyn−/− myotubes expressing wild-type rapsyn-EGFP (**A** and **B**), N88K-rapsyn-EGFP (**C** and **D**) or L361R-rapsyn-EGFP (**E** and **F**) were treated with agrin overnight and then either labelled with α-BuTx-TRITC (**A**, **C** and **E**) or washed for 5 h to remove the agrin before labelling (**B**, **D** and **F**). Magnification: 40×, bar = 20 μm. (**G**) Myotubes were visualized and the number of clusters in 40 fields were counted and expressed as average number of clusters/field.

very different effects. TPR domains mediate protein–protein interaction (Blatch and Lassle, 1999). Rapsyn has a central region made up of multiple TPR domains, and deletion mapping identified TPR1 and TPR2 (domains as shown in Fig. 1) as the most important for rapsyn self-clustering. In addition, deletion mutants of these domains were found to have a dominant negative effect on rapsyn self-clustering when co-expressed with wild-type rapsyn (Eckler *et al.*, 2005). R91L, located in TPR2, was the only one of the mutations we analysed that had an affect, as predicted (Ramarao *et al.*, 2001; Eckler *et al.* 2005), on rapsyn self-association. This single missense substitution dramatically inhibited rapsyn–rapsyn oligomerization, which, theoretically, should still be able to occur via any of the other six TPR domains. However, in contrast with the deletion experiments (Eckler *et al.*, 2005), we did not obviously detect a dominant negative effect of R91L-rapsyn when co-expressed with wild-type rapsyn-DsRed monomer (data not shown); moreover, in patients reviewed at the Oxford Myasthenia Clinic we have not identified a pedigree with rapsyn mutations in which there is a dominant inheritance pattern. However, the possibility of dominant rapsyn mutations should not be ruled out.

Deletion of lysine at position 373 reduces the levels of rapsyn in cell culture. Reporter gene assays indicate that mutations in the *RAPSN* promoter may cause congenital myasthenic syndromes through reduced expression of rapsyn mRNA (Ohno *et al.*, 2003), and consequently reduced rapsyn expression. The association of rapsyn with AChR leads to metabolic stabilization of AChR in the post-synaptic membrane (Wang *et al.*, 1999), and an increased ratio of rapsyn to AChR is thought to enhance AChR stability (Gervasio and Phillips, 2005; Losen *et al.*, 2005). It is not clear whether reduced K373del-rapsyn causes AChR deficiency through an effect on the clustering process itself or through instability of formed or partially formed clusters.

The A25V-rapsyn mutation has a profound effect on AChR clustering. In TE671 cells and rapsyn−/− myotubes, the mutant rapsyn was not present at the membrane and rarely colocalized with the AChR. When A25V-rapsyn-EGFP was expressed in rapsyn−/− myotubes agrin-induced clustering was almost completely abrogated. Another rapsyn mutation close to the N-terminus, L14P, reported previously (Ohno *et al.*, 2002), gave a similar expression pattern (data not shown). A possible explanation for the action of these mutations is an effect on myristylation and thereby binding to the plasma membrane. Alternatively, the rapsyn N-terminal region may be crucial for trafficking to the cell surface, or L14P and A25V may disrupt rapsyn–AChR interactions. However, previous reports have postulated that the coiled-coil domain (residues 298–331) is the major region responsible for these interactions (Bezakova and Bloch, 1998; Ramarao *et al.*, 2001).

Studies of mutations N88K and L361R suggest that they affect several aspects of rapsyn function. Both N88K-rapsyn and L361R-rapsyn showed similar functional characteristics in our expression systems, although lower levels of L361R-rapsyn were observed following transfection into TE671 cells due to either reduced L361R-rapsyn expression or stability. In TE671 cells both mutations led to an ∼50% reduction in rapsyn colocalization with AChR, and both formed reduced numbers of large aggregates of clusters. A decrease in colocalization of N88K-rapsyn-EGFP and AChR in HEK 293 cells was observed previously (Ohno *et al.*, 2002). In rapsyn−/− myotubes the number of agrin-induced AChR clusters, compared with wild-type, was decreased by ∼30% (N88K) or

Table 2 Summary of results

Mutation	Rapsyn cluster formation in TE671 cells	Rapsyn stability	Colocalization with AChR	Agrin-induced AChR clusters (no. per field)	Agrin-induced clusters after washing	Primary defect
WT	Good	Good	>50%	13	Still present	Normal
R91L	Reduced	n.d.	n.d.	n.d.	n.d.	Poor rapsyn self-association
K373del	Good	Severely reduced	n.d.	n.d.	n.d.	Instability of rapsyn
A25V	Good	Reduced	10%	<1	n.d.	Poor rapsyn–AChR interaction
N88K	Good	Good	25%	9	Severely reduced	Unstable AChR clusters
L361R	Good	Reduced	30%	7	Severely reduced	Unstable AChR clusters

60% (L361R). However, when agrin was withdrawn, a dramatic decrease in the number of clusters compared with wild-type was observed, indicating that clusters incorporating these two mutations are unstable.

A significant association with truncating mutations in the second allele and arthrogryposis has been noted (Beeson *et al.*, 2005), suggesting that the lack of function of the second allele may influence disease phenotype. Many factors, including age and the fluctuating course of the disorder, make it very difficult to obtain an objective measure of disease severity. We applied the MG-ADL score to children as well as adults as a simple symptom-based questionnaire as a means of assessing disability when the patient is in a stable phase (Table 1). In our cases we found significantly higher MG-ADL scores in the patients with the functionally more disruptive mutations A25V, R91L and K373del ($P = 0.024$, Mann–Whitney), even when including N88K homozygous Patients 11 and 12 where MG-ADL scores were likely to be increased by co-morbid conditions (Patient 11 also has Perthe's disease and both Patients 11 and 12 had a body mass index of ~40).

Other anecdotal evidence suggesting an involvement of the second mutation with disease severity is that we have not identified deaths in siblings in kinships where the affected child is homozygous for N88K; none of the patients homozygous for N88K have required 3,4-diaminopyridine in addition to their anticholinesterase treatment, implying either that their disease may be less severe or that they respond better to anticholinesterase medication; and four out five of our late-onset cases of AChR deficiency due to rapsyn mutations are homozygous for N88K.

We dissected the pathogenic mechanisms of five naturally occurring rapsyn mutations. The data support the hypothesis that dysfunction in the second mutation of a heteroallelic N88K-rapsyn patient is an important determinant of disease severity. It is tempting to speculate that the underlying cause of the fever-induced severe apnoeic attacks associated with rapsyn mutations is increased N88K-rapsyn-AChR-cluster instability. A raised body temperature might lead to an increase in both protein metabolism and membrane fluidity, thus compromising the structural integrity of the already unstable AChR clusters. Finally, the similarity in functional characteristics of N88K-rapsyn and L361R-rapsyn revealed in this study suggests that there are likely to be patients with AChR deficiency that are either homozygous or heteroallelic for non-N88K mutations within the *RAPSN* coding region.

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