ONLINE MUTATION REPORT

Clusters of non-truncating mutations of P/Q type Ca^{2+} channel subunit $\text{Ca}_v2.1$ causing episodic ataxia 2

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Episodic ataxia type 2 (EA2, MIM 108500) is one of three allelic disorders due to mutations of the *CACNA1A* gene coding for the $\text{Ca}_v2.1$ subunit of P/Q type voltage gated Ca^{2+} channels. The other two allelic diseases are familial hemiplegic migraine (FHM, MIM 141500) and spinocerebellar ataxia type 6 (SCA6, MIM 183086). EA2 is characterised by a complex and highly variable phenotype, widely overlapping that of SCA6.^{1,2} Its main features are episodes of vertigo or ataxia of variable duration and frequency, a permanent cerebellar deficit of variable severity, sometimes progressive, and a cerebellar atrophy typically starting from the anterior portion of vermis. Recently dyskinesia,³ muscular weakness,⁴ and epilepsy⁵ have been described in association with EA2. FHM, on the other hand, is characterised by migraine attacks preceded by symptoms such as unilateral limb paresis or paralysis, paraesthesias, and dysphasia. Interictal cerebellar signs are reported in about 50% of patients.⁶

The *CACNA1A* gene product is the pore forming subunit of P/Q type Ca^{2+} channels, expressed in the brain and particularly in cerebellar Purkinje and granule cells, as well as in neuromuscular junctions.^{7,8} The protein is predicted to have four domains or repeats, each formed by six transmembrane hydrophobic segments (fig 1). Segments S5 and S6 of each domain line the pore region. The S5–S6 linkers are highly conserved sequences folded to leave their extremes in the extracellular space, and place within the pore a P sequence which exerts a critical role for ion selectivity and permeation of the Ca^{2+} channel.⁹

Point mutations in the *CACNA1A* gene are responsible for EA2 and FHM, whereas small expansions of a CAG repeat at the 3' end of the gene characterise SCA6. FHM patients carry exclusively missense mutations.^{10–14} EA2, on the other hand, is reported in 17 cases^{5, 14–20} as due to mutations truncating or severely disrupting (TR) the protein sequence, and in seven cases^{3, 4, 16, 19, 21–23} to non-truncating/disrupting mutations (NTR), either missense or small in-frame deletions. Both types of mutations lead to a loss of function, either complete^{3, 5} or partial.^{4, 24} An EA2 phenotype was also found in association with an expanded 20 CAG allele of the *CACNA1A* gene²⁵ and with a point mutation of the auxiliary β_4 subunit of the same P/Q Ca^{2+} channel, coded by gene *CACNB4*.²⁶

The present work, by substantially expanding the number of EA2 NTR mutations of $\text{Ca}_v2.1$ subunit, shows their tendency to cluster in specific protein regions, and analyses their clinical phenotype in comparison with the phenotype associated with TR mutations.

METHODS

Subjects

The screening for *CACNA1A* mutations was performed overall for 27 index patients and 31 affected relatives; 19 were index patients with a clinical diagnosis of EA2, collected from

Key points

- Episodic ataxia type 2 (EA2) is mostly due to loss of function mutations that truncate or severely disrupt the pore forming ($\text{Ca}_v2.1$) subunit of P/Q type Ca^{2+} channels, coded by the *CACNA1A* gene. Gain of function missense mutations of the same gene are responsible for familial hemiplegic migraine. In a few cases, EA2 is due to mutations that do not truncate or disrupt the $\text{Ca}_v2.1$ subunit.
- Screening for *CACNA1A* gene mutations was carried out for 27 patients with either typical EA2 or cerebellar ataxia of no known genetic type.
- Five new $\text{Ca}_v2.1$ non-truncating/disrupting mutations were detected. From almost doubling the number of these mutations, it clearly emerges that they have a preferential location in specific protein regions, namely S5–S6 linkers and their borders. Their associated clinical phenotype is comparable with that reported for carriers of truncating/disrupting mutations, but data suggest a possible difference in age at onset and frequency of mental retardation.
- The results show that EA2 mutations that do not truncate or disrupt the $\text{Ca}_v2.1$ subunit are not as rare as previously thought. Their associated phenotype might be less severe than that of truncating/disrupting mutations. They are clustered in highly conserved protein regions which may be particularly vulnerable to amino acid changes and are likely to have a critical role in the channel gating activity.

different Italian centres and from the National Hospital, Queen Square, London. All fulfilled minimal diagnostic criteria, including episodes of ataxia or vertigo of variable duration, interictal nystagmus, and absence of mutations in SCA1, 2, 3, 6, 7, 8, 10, 12, 14, 17, and DRPLA genes. The response to acetazolamide was not included among the criteria, since in some cases the burden of the disease was so mild that the side effects of treatment would not compensate its advantages. Nonetheless, most patients (14/19) were treated with acetazolamide and have responded positively in terms of frequency and severity of attacks. Of the 19 EA2 patients, eight were sporadic cases and 11 had, overall, 20 first degree relatives with a similar disorder. These, appropriately informed, consented to participate in the research. Since progressive ataxia is one of the features of EA2,^{1, 21} a

Abbreviations: EA2, episodic ataxia type 2; FHM, familial hemiplegic migraine; SCA6, spinocerebellar ataxia type 6; (N)TR, (non-)truncating/disrupting; SSCP, single strand conformation polymorphism

further eight index patients were selected from our series of 61 cerebellar ataxia (CA) patients who did not carry SCA 1, 2, 3, 6, 7, 8, 10, 12, 14, 17, or DRPLA mutation. Selection was based on having a predominantly vermician cerebellar atrophy and/or a history of vertigo/ataxia episodes preceding the onset of a permanent ataxia, or of fluctuations of their cerebellar symptoms. Two of the eight CA patients were sporadic cases, and six had 11 first degree relatives with vertigo episodes and nystagmus or permanent ataxia, who consented, after appropriate information, to participate in the research.

Mutation screening

Genomic DNA was isolated from peripheral blood leucocytes using standard procedures. All 49 exons of the *CACNA1A* gene and their intron boundaries were amplified by PCR using 55 primer pairs; 54 of these were previously described: exons 1–46 and 47 short form, by Ophoff *et al.*¹⁴; exons 37A, 42, and 47 long form by Jodice *et al.*²⁵ and exon 37B by Trettel *et al.*²⁷ In addition, a primer pair (31B For 5'-AACACGCCTCCCCAA CTG-3'; 31B Rev 5'-GGAGATGCGTTCACAGTTAATG-3') was designed on the basis of NT_031915 contig (actually NT_011295), containing the *CACNA1A* gene, to amplify an alternatively spliced little exon, coding for only two amino acids (NP) and possibly responsible for P or Q specificity of the channel.²⁸ The 14 *CACNB4* gene exons and their boundaries were amplified by PCR using primer pairs previously described for exons 3, 6, 9, 12, 13,²⁶ and newly designed for the other exons (web appendix).

Screening for mutations was performed by single strand conformation polymorphism (SSCP) analysis. Denatured PCR products were electrophoresed on Gene Gel Excel 12.5/24 by GenePhor Electrophoresis Unit (Pharmacia Biotech, www.bio-itworld.com). Conformers were revealed by the silver staining method PlusOne (Pharmacia Biotech). PCR products of patients with atypical migrating bands were sequenced by the MWG BIOTECH Sequencing Service (www.mwg-biotech.com), and checked for cosegregation with the disease, whenever affected relatives were available. The presence of the mutations in the general population was tested by SSCP analysis in 65 randomly selected normal subjects.

RESULTS

Mutation analysis

Of the 27 index cases from the two samples (EA2 and CA), none was carrying *CACNB4* mutations and six were carriers of *CACNA1A* mutations (table 1). Four mutations (1, 2, 3, 4)

were found in the EA2 and two (5, 6) in the CA group. Family 1 in table 1 with mutation F1491S was previously reported³ and will not be further considered, except with regard to the frequency of detected mutations among our patients. All mutations were carried by more than one affected family member, except for index case 8 (R2136C), an adopted person with no traceable parents. All were affecting highly conserved amino acids (fig 2) and none of them was present in 130 random chromosomes. Three of the four missense mutations (table 1) are predicted to induce a drastic change in amino acid characteristics: they lead to exchanges between arginine (R), a large positively charged amino acid, and cysteine (C) or glycine (G), which are small uncharged residues, the first of which possibly is involved in the S–S bond. Only one mutation (V1494I) substituted a highly conserved amino acid with one having a higher molecular weight but similar characteristics. The remaining mutation was an in-frame deletion of two amino acids (Δ MS1488/89) as a result of a six nucleotide deletion.

Figure 1 shows the locations of the five mutations and of seven previously reported EA2 NTR mutations. From almost doubling the number of NTR mutations, it clearly emerges that they are preferentially located in the S5–S6 linkers (9/12), and particularly of domain I and III (8/12), where they tend to cluster at the extremities and their borders. Except for E1757K, they do not directly affect the P sequence (fig 2) responsible for ion selectivity and permeation into the cell,²⁹ nor other functionally known sequences, such as the EF-hand motif in the extracellular portion of III S5–S6 (fig 2), thought to cooperate with the P sequence in selectively binding Ca^{2+} ions.³⁰ They do not affect, either, the residues involved in channel inactivation (not shown in fig 2) in the S5–S6 repeat I³¹ or the S6 repeat III.³²

Three mutations are located outside these preferential regions. The first one (Δ Y1594;A1593D) in IV-S1 is associated with a very mild episodic phenotype without permanent signs.¹⁶ The second one (R1662H), without description of the associated phenotype, is located in IV-S4.²³ The last one (R2136C), carried by one of our EA2 patients with a typical EA2 phenotype, was located in the COOH tail upstream of the polyglutamine repeat.

Clinical features

The main clinical features of the 11 patients carrying the mutations identified in the present study are shown in table 2. Their clinical picture was compared with that described for carriers of TR mutations in several studies (table 3). No statistical comparison, however, was attempted,

Figure 1 Predicted structure of the Ca_v 2.1 subunit of Ca^{2+} channels type P/Q and sites of non-truncating/disrupting (NTR) mutations causing EA2.

Figure 2 Sequence (in single letter code) of the four S5–S6 linkers of human Ca_v 2.1 and their alignment with the same segments of other species and of other human Ca_v 2⁺ channels. P-sequences with ion permeation and selectivity function^{29, 40} are in bold type. EF-hand-motif in repeat III³⁰ is in bold italic type. The residues mutated in EA2 patients are in bold type, enlarged and underlined. Mice mutations *tg* and *rkr* are in bold type, enlarged and underlined for the mouse sequence only. The sequences of domain III include also the initial seven residues of the S6 region. Sequences are from Swissprot Database: Human (HUM) Ca_v 2.1 ID O00555, Mouse (MOU) Ca_v 2.1 ID P97445, Rabbit (RAB) Ca_v 2.1 ID P27884, Drosophila (DRO) Ca_v 2.1 ID P91645, Human (HUM) Ca_v 2.2 ID Q00975, Human Ca_v 2.3 ID Q15878.

since data from different studies might be collected and reported according to differing methods and criteria. The comparison will, therefore, provide only a very rough indication of the overall clinical picture.

Tables 2 and 3 show that the age at onset of our patients was on average at 17 years (95% confidence interval from 12 to 22). This is very similar to the mean (16 years) of 11 previously reported NTR patients^{3, 4, 19, 21, 22} and higher than that in the TR group (mean = 9). In addition, the data confirmed that vertigo/ataxia episodes are not always present in EA2 patients, and the rate of patients without episodes was similar in the two groups. Three of our patients (5-mother, 6-proband, and 6-father), all from families not originally diagnosed as EA2, reported no episodes. Two of them experienced, however, paroxysmic exacerbations of an otherwise slowly progressive ataxia. The type and frequency

of symptoms during the episodes are comparable in the two groups of patients. Headache, including migraine, was a fairly common feature in both groups. Migraine, according to international diagnostic criteria,⁶ was present in four of our patients, only occasionally coinciding with vertigo/ataxia episodes. Accurate interviews with these patients allowed us to exclude any aura in three patients, and the fourth reported a visual aura (flickering lights) without limb weakness or paralysis, paraesthesias, or dysphasia. Acetazolamide treatment was tried consistently in only five cases from the group with an original diagnosis of EA2. A positive response to acetazolamide, in terms of decrease of episode duration and frequency, was obtained whenever tried. The treatment was slightly more efficient in our patients than in carriers of TR mutations, but the sample sizes were too small to provide a meaningful indication.

Mild to severe interictal ataxia was present in a high percentage of patients in both groups. Nystagmus was observed in all of our patients, as expected, being one of the diagnostic criteria. It was most frequently horizontally beating on lateral gaze, but vertical or rotatory nystagmus was observed in 2/11 patients, and in 4/11 it was in all directions of gaze. Cerebellar atrophy at MRI was more frequent in our patients compared with the TR group. This difference, however, might be due to the small sample sizes and/or to the inclusion in our group of CA patients, obviously affected by cerebellar atrophy.

The frequency of the extracerebellar signs (tables 2 and 3) was similar in the two groups of patients, but the type was different. Mental retardation and/or learning difficulty is the most frequent sign among TR carriers, but only one of our patients showed a cognitive deficit (proband 2), and none of the NTR carriers who were previously reported with a

Table 1 Ca_v 2.1 non-truncating (NTR) mutations found in 27 index patients

Family	DNA			Protein		
	ID	n	Nucleotide* Change	Exon	Change	Domain
4	3	1041	TGC-CGC	5	C256R	I S5–S6
	5	2	GGG-AGG	28	G1483R	III S5–S6
	3	2	4739-44	28	ΔMS1488/9	III S6
1†	2	4747	TTC-TCC	28	F1491S	III S6
6	3	4755	GTC-ATC	28	V1494I	III S6
2	1	6681	CGC-TGC	45	R2136C	COOH-ter

*Nucleotide numbers are based on the human CACNA1A sequence (accession number AC X99897). †Family already reported by Guida et al.³ ID, identification; n, number of patients.

Table 2 Clinical features in 11 patients carrying EA2 non-truncating *CACNA1A* mutations

Clinical feature	#2 proband	#3 proband	#3 mother	#4 proband	#4 son	#4 son	#5 proband	#5 son	#6 proband	#6 father	#6 brother
Age (years)	54	32	64	49	17	13	57	28	34	60	38
Age at onset (years)	25	8	15	10	10	10	24	22	28	—	20
Symptoms											
Episodic	+	+	+	+	+	+	fluctuats	+	fluctuats	—	+
Frequency	monthly	weekly	monthly	weekly	weekly	weekly	monthly	yearly	monthly	—	variable
Duration	mins//hrs	hrs	mins/hrs	hrs	hrs	hrs	hrs/days	secs/mins	hrs/days	—	mins
Ataxia	+	+	—	+	+	+	—	—	—	—	—
Dysarthria	+	+	—	+	+	+	—	—	—	—	—
Vertigo	+	+	+	—	—	—	—	+	—	—	+
Nausea/vomiting	+	—	—	+	+	+	—	—	—	—	—
Oscillopsia	—	—	—	+	+	+	—	—	—	—	—
Headache	—	—	—	—	—	—	—	+	—	—	—
Migraine	+	—	—	—	—	—	+	—	+	—	+
Aura	—	—	—	—	—	—	auditory	—	—	—	—
Interictal cerebellar											
Ataxia	mild	mild	mild	mild	—	mild	severe	—	severe	mild	—
Dysarthria	—	—	—	mild	—	—	mild	—	mild	—	—
Dysmetria	—	—	—	—	—	—	mild	—	severe	mild	—
Adiadochokinesia	—	—	—	—	—	—	mild	—	mild	mild	—
Nystagmus	+	+	+	+	+	+	+	+	+	+	+
V atrophy	+	+	+	+	NT	NT	+	NT	+	NT	NT
H atrophy	+	—	—	+	NT	NT	—	NT	+	NT	NT
Extracerebellar	+	—	—	—	—	—	+	—	+	—	—
Acetazolamide response	+	+	NT	+	+	+	NT	NT	NT	NT	NT
Mutation	R2136C	ΔMS 1488/9	ΔMS 1488/9	C256R	C256R	C256R	G1483R	G1483R	V1494I	V1494I	V1494I

Mins, minutes; hrs, hours; secs, seconds; NT, not tested; V, vermis; H, hemisphere; fluctuations, fluctuation of permanent ataxia.

detailed clinical phenotype^{3, 4, 19, 21, 22} showed a cognitive deficit. Proband 2 presented with a presenile mild dementia and cortical-subcortical brain atrophy. Since she underwent a thyroidectomy in the past, the cognitive deficit could be related to hypothyroidism resulting from loose compliance or imperfect control of the replacement therapy.^{35, 36} The other two patients of the NTR group with extracerebellar signs had a sensorineural hypoacusia and a labyrinth areflectivity in one case (fam 5-proband), and a complex ataxo-spastic disorder in the other (fam 6-proband). The deafness of the former patient was of cochlear origin, suggesting the coexistence of Ménière's syndrome (MIM 156000)³³ or

non-syndromic deafness with vestibular dysfunction³⁴ (DFNA9, MIM 601369) which, however, could not account for the patient's cerebellar features. Fam 6-proband presented with an ataxo-spastic clinical picture with exacerbations of her feeling of imbalance. The proband had inherited the ataxo-spastic disease from the mother, presenting with the same picture but without exacerbations, and the EA2 mutation from the father, who showed mild EA2 signs, as did the proband's brother. The mild EA2, paternally inherited phenotype in the proband was probably obscured by the maternally inherited disorder, except for the fluctuations of the cerebellar symptoms.

Table 3 Frequency of main clinical features in carriers of Ca_v2.1 truncating/disrupting (TR) mutations reported in the literature, and comparison with those found in carriers of non-truncating/disrupting (NTR) mutations in the present work

	Patients with Ca _v 2.1 mutations										
	TR								NTR		
Features	Ref ¹⁵	Ref ^{16†}	Ref ¹⁷	Ref ⁵	Ref ¹⁸	Ref ¹⁹	Ref ²⁰	Total†	%	Our study	%
Patients (number)	1	20	15	1	2	3	1	43		11	
Families (number)	1	6	1	1	2	3	1	15		5	
Age*	38	40	36	?	36	31	41	38		41 ± 3	
Age at onset (range)*	1(1)	10(2–19)	10(8–15)	8(8)	1.5(1.5)	3.5(2–5)	10	9(1–19)		17 ± 2(8–28)	
Episodes	1/1	16/20	?	1/1	2/2	3/3	1/1	24/28	0.8	8/11	0.7
ataxia	1/1	13/20	?	1/1	2/2	3/3	1/1	21/28	0.7	5/11	0.5
NV	1/1	9–10/20	?	0/1	1/2	2/3	1/1	14–15/28	0.5	4/11	0.4
Vertigo	0/1	7–8/20	?	0/1	2/2	1/3	0/1	10–11/28	0.4	5/11	0.5
VA‡	0/1	4/20	?	1/1	2/2	1/3	0/1	8/28	0.3	3/11	0.3
Headache	0/1	6–7/20	11/15	1/1	2/2	1/3	0/1	21–22/43	0.5	5/11	0.5
Dysarthria	1/1	8–9/20	?	0/1	1/2	0/3	0/1	10–11/28	0.4	5/11	0.5
Response to acetazolamide	1/1	?	7/8	NT	1/2	2/3	1/1	12/15	0.8	5/5	1.0
Interictal signs											
Cerebellar ataxia	1/1	12/19	10/14	1/1	1/2	2/3	0/1	27/41	.7	8/11	0.7
Nystagmus	1/1	13/19	13/15	1/1	2/2	2/3	1/1	33/42	0.8	11/11	1.0
Atrophy at MRI	0/1	2/4	3/3	0/1	?	?	0/1	5/10	0.5	6/6	1.0
Extracerebellar	0/1	10/20	0/15	1/1	0/2	1/3	0/1	12/43	0.3	3/11	0.3
Cognitive	0/1	9/20	0/15	1/1	0/2	1/3	0/1	11/43	0.3	1/11	.1

Only studies with a detailed description of the clinical phenotype were taken into consideration. *Mean ± SE in years; †the exact number of patients showing a specific symptom could not always be reconstructed, this particularly referring to one patient and the frequency with and without him/her is reported; ‡VA, visual anomalies include oscillopsia, blurred vision, dyslopia; NT, not tested; ?, data not reported; NV, nausea and vomiting.

DISCUSSION

A screening for *CACNA1A* and *CACNB4* mutations was performed in two groups of index patients, one with EA2 and the other with CA of unknown genetic type. Mutations were found only in the *CACNA1A* gene: overall, six mutations, all of NTR type, were found in 27 index patients (one mutation of this group of patients was already reported by Guida *et al*³). Such a low detection rate has already been reported in EA2 studies¹⁴ and might be due either to genetic heterogeneity, or to phenocopies, or to mutations in the still unexplored portions of the *CACNA1A* gene, such as the expression regulatory regions.

The main cerebellar features, both paroxysmic and interictal, in 11 carriers of these NTR mutations were comparable with those previously reported in EA2 patients with TR mutations. The comparison, however, suggests that the former might have an earlier onset and a more frequent cognitive deficit than the latter. The differences are maintained also when the previously reported NTR patients^{3,4,19,21,22} are considered. At present, however, these are just indications of possible trends differentiating the two phenotypes, which must be confirmed by more accurate and systematic assessments in a much larger patient population.

The present and previous EA2 NTR mutations appear to be located at preferential sites of Ca_v2.1 protein, since most of them cluster at the S5–S6 linkers and their borders, outside the sequences with a known specific function. When compared with FHM missense mutations, which are widely scattered along S4, S5, S6, and their linkers in all 4 domains, this appears to be a quite distinct feature of EA2 mutations. Only two FHM mutations are in S5–S6 linkers, and are in the selectivity filter sequence: T666M in domain II, associated with an ataxic phenotype,¹² and V1457L in domain III, with no details of the associated clinical picture.¹¹ Furthermore, it should be noted that two out of three NTR *CACNA1A* mutations carried by ataxic mice, tottering (*tg*) and rocker (*rkr*), have a location similar to that of human NTR mutations, namely *tg* in S5–S6 linker of the II repeat³⁷ and *rkr* in S5–6 linker of the III repeat³⁸ (fig 2).

Of the three EA2 mutations located outside the preferential areas, one (R2136C, identified in the present study) is in the COOH tail upstream of the polyglutamine repeat. It is noteworthy that a nearby mutation was detected in a patient with a cerebellar deficit (P Giunti, unpublished data), possibly indicating the site of a further cluster. The R2136C mutation does not affect the binding sites for calmodulin and auxiliary β subunits present in this protein region.²⁸ It might, however, affect the state dependent mobility of the COOH tail—that is, its ability to cooperate in channel activity by conformational changes,³⁹ particularly in inactivation gating.³² The change from arginine to cysteine, probably involved in S–S bonds, could limit the conformational mobility of the C-terminus of the protein.

Four of the previously reported NTR mutations have been functionally tested, and all were predicted to induce a loss of channel function. F1491S was shown by Guida *et al*³ to completely abolish the ion flux into HEK 293 cells, and in the study by Jen *et al*⁴ F1406C appeared to decrease the current density, leaving a small influx of divalent ions in COS7 cells. G293R was shown to shift the current voltage relationship toward more positive potentials and enhance inactivation in *Xenopus* oocytes, as did mutation Δ Y1594;A1593D.²⁴ Although no functional data are yet available for the remaining mutations, their position in specific protein regions strongly supports their functional relevance. It appears highly unlikely that functionally irrelevant mutations, associated with the same phenotype, will be found in clusters that include mutations known to alter the channel function.

Recently, Jiang *et al*⁴¹ proposed a new model, based on a protein structure analysis, of how membrane voltage gates the pore in K⁺ channels. According to the model, the membrane depolarisation induces S3 and S4 segments to move within the membrane, from a position almost perpendicular to the pore and near the intracellular surface, to a position parallel to the pore near the extracellular surface. The displacement of S4 opens the channel by pulling S5 and S6 away from the axis of the pore. Should this model hold also for Ca²⁺ channels, considering that voltage gated channels share a common structure, this would imply that S5–S6 linkers might have a highly relevant role in transmitting the correct gating movement from S5 to S6. Mutations in the S5–S6 regions, hence, could interfere with channel gating activity.

In conclusion, the present results show that EA2 NTR mutations are not as exceptional as previously thought. Their associated phenotype is likely to differ from that of TR mutations by delaying age at onset and preventing cognitive deficit. Their large majority clusters in at least two main protein regions, providing indirect evidence of their functional relevance and indicating that these mutational hot spots deserve a deeper analysis of their role in the channel activity.

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