Large-scale calcium channel gene rearrangements in Episodic Ataxia and Hemiplegic Migraine

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Abstract

Background: Episodic ataxia type 2 (EA2) and familial hemiplegic migraine type 1 (FHM1) are autosomal dominant disorders characterized by paroxysmal ataxia and migraine respectively. Point mutations in \textit{CACNA1A}, which encodes the neuronal P/Q-type calcium channel, account for many cases of EA2 and FHM1. The genetic basis of cases without \textit{CACNA1A} point mutations is not fully known. Standard DNA sequencing methods may miss large scale genetic rearrangements such as deletions and duplications. We investigated whether large-scale genetic rearrangements in \textit{CACNA1A} can cause EA2 and FHM1. Methods: We used multiplex ligation-dependent probe amplification (MLPA) to screen for intragenic \textit{CACNA1A} rearrangements. Results: We identified five previously unreported large scale deletions in \textit{CACNA1A} in seven patients with EA and in one case with hemiplegic migraine. One of the deletions (exon 6 of \textit{CACNA1A}) segregated in nine affected EA individuals over four generations of a family previously mapped to 19p13. In addition, we identified the first pathogenic duplication in \textit{CACNA1A} in an index case with isolated episodic diplopia without ataxia and in a first degree relative with EA2. Conclusions: Large-scale deletions and duplications can cause \textit{CACNA1A} associated channelopathies. Direct DNA sequencing alone is not sufficient as a diagnostic screening test.
**Introduction**

Episodic ataxia type 2 (EA2) and familial hemiplegic migraine type 1 (FHM1) are dominant disorders caused by mutations in *CACNA1A* on chromosome 19p1.3 [1, 2]. This gene encodes the alpha subunit of the P/Q-type calcium channel [3], which is the principal channel involved in neurotransmission at central nerve terminals [4]. Episodic ataxia type 2 (EA2) is characterized by paroxysmal attacks of cerebellar dysfunction resulting in incoordination, slurred speech, ataxic gait and vomiting [5-7]. Epilepsy has also been reported as part of the phenotype in some cases [8, 9]. Familial hemiplegic migraine type 1 (FHM1) is a variant of migraine with aura resulting in attacks of unilateral hemiplegia and/or hemisensory loss [10]. Nonsense and missense mutations, which result in a premature stop codon or aberrant splicing, account for many cases of EA2 cases [11-13] while missense mutations altering conserved amino acid residues tend to associate with FHM1 [14] but also cause EA2 [15].

A significant proportion of patients with typical clinical features of EA2 do not have point mutations in *CACNA1A*, suggesting genetic heterogeneity. Mutations in two additional genes (*CACNB4* and *SLC1A3*) have been reported in a small number of EA2 patients. A mutation in *CACNB4*, encoding the beta subunit of the P/Q-type calcium channel has been described in one family with an EA2 type phenotype (termed EA5) and also in another family with juvenile myoclonic epilepsy [16]. *SLC1A3*, encoding the excitatory amino acid transporter EAAT1 has been associated with EA2 in three families (termed EA6) [17, 18]. Two additional genes have been associated with FHM: *ATP1A2* [19-21] and *SCN1A* [22] cause FHM types 2 and 3 respectively. However, many patients with typical features of EA2 and FHM1 do not have point
mutations in these genes. Together, these observations suggest that either mutations in other
genes are responsible for EA2 and FHM1 or that they are due to uncharacterized genetic defects
in *CACNA1A*, which are not detected by standard mutation screening strategies, such as those in
introns, alternatively spliced exons and regulatory regions.

In the present study, we used multiplex ligation dependent probe amplification (MLPA) to screen
for large scale genetic rearrangements in the *CACNA1A* gene in a series of patients who had
clinical EA or FHM, but in which no mutations in *CACNA1A* were detected by direct DNA
sequencing. Our aim was to investigate the extent to which large deletions and duplications in
the gene account for these disorders.

**Methods**

*Patients.* Ethical approval was obtained from the UCLH ethics committee. Patient samples were
referred from England, Republic of Ireland and Denmark. Critical evaluation of patient data was
carried out in accordance with published criteria [5, 6].

*MLPA Analysis.* The MLPA *CACNA1A* test kit (SALSA P279_A1) is manufactured by MRC-
Holland, Amsterdam. MLPA probes for exons 1, 4, 6-8, 10, 11, 13, 16, 18, 20, 24, 25, 27, 29, 31,
33, 35, 38, 39, 40, 43, 46 and 47 of CACNA1A. MLPA was performed according to
manufacturers’ instructions and the amplified fragments were analysed using an ABI 3730xl
capillary sequencer (Applied Biosystems) and the GeneMarker v1.70 software (SoftGenetics).
Individual peaks corresponding to each exon (*CACNA1A* as well as control genes) are identified
based on the difference in migration relative to the size standards (LIZ 500, Applied
Biosystems). The GeneMarker analysis software uses the MLPA ratio method to determine the deviation of each allele peak, relative to the average deviation of all peaks. This method standardises the data so that the median point within the data set is considered to be 1 and calculates the deviation of each peak from this median as a ratio. A peak with a ratio of less than 0.75 indicates a deletion while a peak with a ratio of greater than 1.25 indicates duplication.

**DNA Sequencing.** Automated direct DNA sequencing was performed on PCR products using primers that amplified each exon of *CACNA1A*. Samples were sequenced (bidirectional) using the ABI Big Dye Terminator Sequencing Kit version 1.1, M13 universal primers on an ABI 3730xl capillary sequencer and analysed using SeqScape v2.5 sequencing analysis software (Applied Biosystems).

**Results**

Eight unrelated patients were found to have rearrangements in *CACNA1A*. Six had a diagnosis of EA2, one had sporadic hemiplegic migraine and one patient had episodic diplopia. In three patients the data indicated that at least a single exon deletion was present, while in 4 patients, analysis indicated a deletion involving multiple exons. One patient had a single exon duplication (See Table 1 for summary of clinical and genetic results, Figure 1 and Figure 2). All deletions and the single duplication were absent in a panel of 100 control chromosomes.
Patient 1

Episodes of ataxia began at the age of 39 years with stereotyped attacks of unsteadiness and dysarthria lasting a few hours. There were no precipitants for attacks but sleep helped their resolution. The attacks were completely abolished by acetazolamide. Interictal examination revealed square wave jerks, gaze-evoked nystagmus and jerky pursuits, but no limb ataxia. Family history indicated dominant inheritance with the proband’s daughter and father also affected (Figure 3A). The daughter reported stress-induced attacks of unsteadiness, dysarthria and nausea which lasted several hours. She responded well to acetazolamide. Examination revealed gaze-evoked nystagmus. The proband’s father is deceased but had a history consistent with EA2.

MLPA identified a heterozygous deletion using the probe for exon 4 of \textit{CACNA1A} in the proband and in his daughter (Figure 1B). Probes for exons 1 and 6 gave normal results, indicating that the maximum length of the deletion is 146131 base pairs. Exon 4 encodes amino acids 181 to 211 of the \textit{CACNA1A} protein, which includes the last 4 amino acids of S3, the extracellular linker between S3 and S4, the entire S4 segment, (amino acids 191 to 209) and the first 11 amino acids of the cytoplasmic linker between S4 and S5 of the first domain of the protein.

Patient 2

The proband was from a family with nine affected individuals, spanning four generations exhibiting a classical phenotype of EA2 (Figure 3B). She developed attacks from the age of four years. These consisted of diplopia, slurred speech, unsteadiness and headaches. Excitement,
stress and tiredness triggered episodes. Examination at the age of 59 years revealed cerebellar signs including gaze-evoked nystagmus, cerebellar dysarthria, finger nose ataxia and a marked broad based gait. Since the age of 30 there has been persistent gait ataxia and dysarthria.

Although the age of onset and disease severity varied amongst the other affected family members, all experienced similar symptoms to that of the proband. All affected members showed a good response to acetazolamide.

Linkage analysis identified a common haplotype at 19p13 that segregated with disease status in the family (data not shown). However, DNA sequencing of the entire coding and flanking regions of \textit{CACNA1A} in the proband did not identify a point mutation. In contrast, MLPA identified a heterozygous deletion using the probe for exon 6 of \textit{CACNA1A} in the proband (Figure 1C). There was no probe for exon 5, but exons 4 and 7 were present. Based on this data, the maximum size of the deletion is predicted to be 35778 base pairs. Exon 6 encodes amino acids 263 to 326 of the \textit{CACNA1A} protein, which forms part of the extracellular linker between S5 and S6 of domain I. The deletion was confirmed to segregate with the EA2 phenotype and was found in eight affected family members and was absent in one unaffected family member.

\textbf{Patient 3}

Patient 3 described lifelong “spasms” of his eyes by which he meant attacks of double vision and oscillopsia which occurred up to twice a day and lasted one to two hours. These attacks were often triggered by sudden head movement. He denied other motor or sensory symptoms. There was no history of ataxia but he did report dizziness which occasionally accompanied his attacks.
of double vision. On examination he had gaze-evoked nystagmus. There were no cerebellar signs in his limbs and no gait ataxia. MRI brain was normal and he did not respond to acetazolamide. His father had excitement-induced attacks of ataxia and slurred speech which would last one hour, during which he reportedly had oscillopsia and nystagmus.

MLPA identified a heterozygous duplication using the probe for exon 6 of the CACNA1A gene in the proband (Figure 1D). The maximum length of the duplication is predicted to be 35778 base pairs. As previously described, exon 6 encodes amino acids 263 to 326 of the CACNA1A protein which forms part of the extracellular domain between S5 and S6 of repeat 1. It was not possible to obtain DNA from his father.

Patient 4

Patient 4 experienced stereotyped attacks of EA2 from the age of 5 years. Episodes consisted of vertigo, slurred speech and incoordination in her arms and legs. A throbbing headache and vomiting accompanied half her attacks which lasted from a few hours to three days. Sleep often either aborted or lessened the severity. Interictally, she described occasional oscillopsia. There was no family history of episodic ataxia or migraine. When examined at 18 years, she had square wave jerks in the primary position with gaze-evoked nystagmus and jerky pursuit movements. There was mild gait ataxia. An EEG demonstrated a mild excess of irregular slow wave bursts with spike wave transients suggestive of epileptiform paroxysms. However, an EEG taken at the time of an attack remained in its normal wake state, although the bursts were a little more frequent. There was no history to suggest seizures. Unusually, there was no beneficial response to acetazolamide.
A heterozygous deletion using the probe for exon 27 was detected by MLPA (Figure 1E). Probes for exons 25 and 29 gave normal results, indicating that the maximum length of the deletion is 7474 base pairs. Exon 27 encodes 1418 to 1463 of the CACNA1A gene which forms part of the extracellular linker between S5 and S6 of domain III.

**Patient 5**

From the age of 18 months, patient 5 experienced attacks of unsteadiness and vomiting which lasted up to 6 hours and occurred twice a month. There was no relevant family history. Interictal examination revealed jerky pursuit movements, gaze-evoked nystagmus and a mild broad-based gait. There was a good response to acetazolamide.

MLPA identified heterozygous deletions of exons 20 to 38 (Figure 1F). As exons 18 and 39 were present in the patient, the maximum length of the deletion is predicted to be 86155 base pairs. This large deletion is predicted to cause a frameshift resulting in a truncated peptide and degradation of the mRNA transcript.

**Patient 6**

Episodes of “clumsiness” began at the age of 10. There was sudden onset of dizziness and the inability to sit or stand and associated nausea. Examination showed reduced balance on heel toe walking and mild dysdiadochokinesis. A the age of 11, she experienced typical absence seizures with ictal EEG recordings consistently showing 3Hz generalized spike and wave discharges. A few months later, she experienced attacks of vertigo, nausea and vomiting. Initially they occurred weekly and would last up to four hours. Sleep would help attack resolution. Examination during
one such episode revealed ataxia, problems with sitting balance (was unable to sit without falling) and gaze-evoked nystagmus. Initial treatment with acetazolamide resulted in a marked improvement although attacks were not completely abolished. Over the years, the attack frequency varied from twice monthly to periods of six months when she was attack free. The duration of the attacks diminished with age. An MRI of the brain at 12 years was normal, although subsequent scans at the age of 16 and 18 years demonstrated slight atrophy of the cerebellum, and in particular the vermis. There was no family history of EA2.

Patient 7
Assessed at age 37 years, there was a 20 year history of frequent episodic neurological symptoms affecting her right side. Attacks comprised of loss of vision in her right hemifield, weakness and numbness of the right side of her face, right arm and leg. These attacks generally lasted between 15 and 30 minutes. The episodes varied in frequency from twice monthly to 3 or 4 times per year. They were usually not accompanied by headaches, nausea, vomiting or photophobia. She also described typical migraine with nausea and vomiting which occurred separately from the hemiplegic attacks, but these were infrequent. Neurological examination was entirely normal as was an MRI brain. The attacks were considered to be consistent with hemiplegic migraine. There were no vascular risk factors. Her mother suffered from migraine, but did not have attacks of weakness or abnormal sensation. Her paternal grandfather was described to have had several strokes in his 40’s but further details were not available.

Patient 8
Attacks of unsteadiness, dizziness and nausea occurred from the age of 2.5 years. These were precipitated by nausea and relieved by sleep and would occur on average once a week. There was no family history of episodic ataxia or migraine. Interictal examination at the age of 8 years revealed mild dysdiadochokinesia with no other cerebellar signs. EEG and brain MRI were both normal. Acetazolamide was not tested.

In patients 6, 7 and 8, MLPA probe analysis was consistent with a heterozygous deletion of exons 39-47 (Figure 1G). As exon 38 was present in both cases, the maximum length of the deletion is predicted to be 18225 base pairs. The absence of multiple exons suggests a likely frameshift resulting in a truncated peptide and degradation of the mRNA transcript.

**Discussion**

To date a large number of point mutations in *CACNA1A* have been reported to cause EA2 and the allelic disorder FHM1 [1, 6, 10, 11, and 14]. However, a significant number of patients with typical EA2 or FHM1 do not harbour point mutations in *CACNA1A*. Sequencing of additional EA2 candidate genes, *SCL1A3* and *CACNB4* failed to identify any causative mutations in some of our cohort [23]. A deletion mutation mechanism has been described in other ion channel genes such as in *SCN1A*, which causes severe myoclonic epilepsy of infancy (SMEI) [24]. Recently, a large deletion in *CACNA1A* was reported in the two affected members of a family with EA2 [25].
In the present study, we have identified \textit{CACNA1A} rearrangements in 8 patients. Although, we have not undertaken fine mapping of these rearrangements to assess which predict translational frameshift, a number of points indicate they are likely to be pathogenic, not least their absence in a panel of one hundred control chromosomes. Their location within the channel protein suggests they would remove or duplicate highly conserved regions of the channel protein. It is probable that the deletion in patient 5 which spans at least exons 20 to 38 and in patients 6, 7 and 8 which removes all exons downstream of exon 38 would result in premature truncation of the channel protein or an mRNA transcript that undergoes nonsense-mediated decay. In patients 1, 2, 3 and 4, in whom deletions of at least one exon have been identified, the fate of the mRNA transcript will depend on whether the mutations affect the reading frame. If inframe, such large rearrangements are likely to interfere with correct protein assembly, folding and trafficking, which are all important for channel function. In patient 1, the deletion spanned exon 4 which encodes the S4 voltage sensor segment of domain I. Removal of the voltage sensor of the channel is likely to have deleterious effects on kinetic parameters such as the voltage-dependence of activation. The segregation of the deletion in patient 1’s family in eight affected individuals and its absence in one unaffected member also supports pathogenicity. Segregation was also present in patient 1’s affected daughter.

The phenotypic spectrum observed in this study included EA2 but also hemiplegic migraine and episodic diplopia. Patient 7 presented with symptoms consistent with hemiplegic migraine but lacking family history, suggesting that deletions can associate with this phenotype and that in this patient it is a new mutation or there is reduced penetrance. Although very small insertion/deletion mutations have been previously described in FHM1 patients [26], large scale
deletions have not been reported. In patient 3, we identified the first exonic duplication in *CACNA1A*. He presented with an episodic eye movement disorder without ataxia or dysarthria. Eye movement abnormalities including diplopia and oscillopsia are documented in EA2 patients but are not commonly reported in isolation. Patient 3’s father was reported to have typical EA2, suggesting phenotypic variability amongst members of the same kindred.

The identification of a deletion in the large family with EA2 (family of patient 2) highlights the importance of screening for deletions in *CACNA1A* as part of the molecular characterization of EA2. Linkage analysis had previously identified a common haplotype at 19p13 that segregated with affected status in the family but sequencing had failed to identify any point mutations or small insertions or deletions in *CACNA1A*. Segregation of the deletion with the disease phenotype supports the view that this deletion is disease-causing.

One limitation of the MLPA analysis is the current unavailability of probes for all exons of the *CACNA1A* gene. Therefore a negative MLPA result in this study does not exclude deletions or duplications of the remaining exons. In addition, fine mapping of the breakpoints of the rearrangements requires further work. The only previously described large deletion was considered to have arisen through homologous recombination of *Alu* sequences [25]. Identification of these repeat sequences in the introns together with MLPA analysis of the remaining exons will narrow down the break point search region. Despite these limitations, the data presented provides important new evidence that large scale rearrangements of *CACNA1A* can associated with episodic ataxia, episodic diplopia and probably with hemiplegic migraine.
The data presented here have expanded the mutation spectrum in \textit{CACNA1A} which has implications for DNA diagnosis. We suggest that screening for large scale rearrangements by techniques such as MLPA should be considered as a first line approach to genetic diagnostic testing for \textit{CACNA1A} associated channelopathies. Sequencing of \textit{CACNA1A} should then be undertaken if no rearrangements are identified. Our findings indicate that large scale gene rearrangements are an important case of \textit{CACNA1A} associated neurological disease.

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\textbf{References}


Figure Legends

Figure 1: MLPA traces showing deletions and duplications of CACNA1A exons. The red peak is a control peak of known copy number to which the patient DNA (blue peak) is compared and a ratio calculated. A peak with a ratio of less than 0.75 indicates a deletion while a peak with a ratio of greater than 1.25 indicates duplication. Numbers correspond to exons in CACNA1A. A: Normal control B: deletion of exon 4 (patient 1; ratio: 0.43); C: deletion of exon 6 (patient 2; ratio: 0.55); D: duplication of exon 6 (patient 3; ratio: 1.39; E: deletion of exon 27 (patient 4; ratio: 0.550; F: deletions of exons 20 (0.54), 24 (0.55), 25 (0.53), 27 (0.53), 29 (0.52), 31 0.48), 33 (0.53), 35 (0.48) and 38 (0.52) seen in patient 5; G: deletions of exons 39 (0.52), 40 (0.54), 43 (0.52), 46 (0.31) and 47 (0.50) seen in patient 6 (a similar trace was detected in patients 7 and 8).

Figure 2: Maximum extent of the deletions and duplication detected using MLPA. Red squares indicate exons for which a probe is included in the MLPA analysis. Letters represent patients as they appear in the main text.

Figure 3: Families of patients 1 (A) and 2 (B). Filled symbols represent affected individuals. Arrows represent the proband.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Dx</th>
<th>Age onset (yrs)</th>
<th>Episodic features</th>
<th>Episode duration</th>
<th>Interictal Examination</th>
<th>FH</th>
<th>Additional features</th>
<th>MRI Brain</th>
<th>Response to ACZ</th>
<th>Exon del/dup</th>
<th>Maximum length of del/dup (bp)</th>
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<tbody>
<tr>
<td>1</td>
<td>EA</td>
<td>39</td>
<td>Ataxia dystartria</td>
<td>Few hours</td>
<td>SWJ, GEN JP</td>
<td>Daughter/father affected</td>
<td>headaches</td>
<td>-</td>
<td>Good</td>
<td>del: exon 4</td>
<td>146131</td>
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<tr>
<td>2</td>
<td>EA</td>
<td>4</td>
<td>Diplopia dystartria ataxia headache</td>
<td>24 hours</td>
<td>GEN; dystartria; ataxic gait</td>
<td>8 affected</td>
<td>Persistent cerebellar syndrome</td>
<td>-</td>
<td>Good</td>
<td>del: exon 6</td>
<td>35778</td>
</tr>
<tr>
<td>3</td>
<td>ED</td>
<td>lifelong</td>
<td>Diplopia oscillopsia</td>
<td>Few hours</td>
<td>GEN</td>
<td>Father had EA</td>
<td>-</td>
<td>Normal</td>
<td>None</td>
<td>dup: exon 6</td>
<td>35778</td>
</tr>
<tr>
<td>4</td>
<td>EA</td>
<td>5</td>
<td>Vertigo dystartria ataxia</td>
<td>Hours to days</td>
<td>SWJ, GEN JP</td>
<td>No</td>
<td>Epileptiform EEG</td>
<td>-</td>
<td>None</td>
<td>del: exon 27</td>
<td>7474</td>
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<tr>
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<td>1.5</td>
<td>Ataxia vomiting</td>
<td>Few hours</td>
<td>GEN, JP Ataxic gait</td>
<td>No</td>
<td>-</td>
<td>-</td>
<td>Good</td>
<td>del: exons 20-38</td>
<td>86155</td>
</tr>
<tr>
<td>6</td>
<td>EA</td>
<td>10</td>
<td>Ataxia vertigo vomiting</td>
<td>Several hours</td>
<td>GEN DDK</td>
<td>No</td>
<td>Absence seizures</td>
<td>Cerebellar atrophy</td>
<td>Initial response</td>
<td>del: exons 39-47</td>
<td>18225</td>
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<tr>
<td>7</td>
<td>SHM</td>
<td>17</td>
<td>LOV in right hemifield right sided hemiplegia</td>
<td>30 mins</td>
<td>Normal</td>
<td>No</td>
<td>-</td>
<td>Normal</td>
<td>Not tried</td>
<td>del: exons 39-47</td>
<td>18225</td>
</tr>
<tr>
<td>8</td>
<td>EA</td>
<td>6</td>
<td>Ataxia vertigo nausea</td>
<td>Few hours</td>
<td>DDK</td>
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<td>-</td>
<td>Normal</td>
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<td>del: Exons 39-47</td>
<td>18225</td>
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Table 1: Summary of clinical and genetic findings in patients with CACNA1A rearrangements detected by MLPA; Dx= Diagnosis; EA= Episodic ataxia; SHM= Sporadic hemiplegic migraine; ED= Episodic diplopia; LOV= loss of vision; SWJ= square wave jerks; GEN= gaze evoked nystagmus; JP= jerky pursuit; DDK= dysdiadochokinesis; FH= family history; del= deletion; dup= duplication; bp= base pairs
Figure 1. MLPA traces showing deletions and duplications of CACNA1A exons. The red peak is a control peak of known copy number to which the patient DNA (blue peak) is compared and a ratio calculated. A peak with a ratio of less than 0.75 indicates a deletion while a peak with a ratio of greater than 1.25 indicates duplication. Numbers correspond to exons in CACNA1A. A: Normal control B: deletion of exon 4 (patient 1; ratio: 0.43); C: deletion of exon 6 (patient 2; ratio: 0.55); D: duplication of exon 6 (patient 3; ratio: 1.39); E: deletion of exon 27 (patient 4; ratio: 0.55); F: deletions of exons 20 (0.54), 24 (0.55), 25 (0.53), 27 (0.53), 29 (0.52), 31 (0.48), 33 (0.53), 35 (0.48) and 38 (0.52) seen in patient 5; G: deletions of exons 39 (0.52), 40 (0.54), 43 (0.52), 46 (0.31) and 47 (0.50) seen in patient 6 (a similar trace was detected in patients 7 and 8).
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