Dramatically different levels of \textit{cacna1a} gene expression between pre-weaning wild type and \textit{leaner} mice

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\textbf{ABSTRACT}

Loss of function mutations of the \textit{CACNA1A} gene, coding for the α1A subunit of P/Q type voltage-gated calcium channel (Ca\textsubscript{V2.1}), are responsible for Episodic Ataxia type 2 (EA2), an autosomal dominant disorder. A dominant negative effect of the EA2 mutated protein, rather than a haploinsufficiency mechanism, has been hypothesised both for protein-truncating and missense mutations. We analysed the \textit{cacna1a} mRNA expression in \textit{leaner} mice carrying a \textit{cacna1a} mutation leading to a premature stop codon. The results showed a very low mutant mRNA expression compared to the wild type allele. Although the mutant mRNA slightly increases with age, its low level is likely due to degradation by nonsense mediated decay, a quality control mechanism that selectively degrades mRNA harbouring premature stop codons. These data have implications for EA2 in humans, suggesting a haploinsufficiency mechanism at least for some of the \textit{CACNA1A} mutations leading to a premature stop codon.

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1. Introduction

The human \textit{CACNA1A} gene on chromosome 19p13 encodes the α\textsubscript{1A} pore-forming subunit of P/Q type voltage-gated Ca\textsuperscript{2+} channel (Ca\textsubscript{V2.1}). The protein has a tissue specific expression in neurons, particularly in cerebellar Purkinje and granule cells, as well as in neuromuscular junctions [1–3]. Mutations of the \textit{CACNA1A} gene leading to a complete or a partial impairment of Ca\textsuperscript{2+} influx [4–8] are responsible for Episodic Ataxia type 2 (EA2, OMIM 108500). EA2 is an early onset, autosomal dominant disorder characterised by attacks of vertigo/ataxia, visual disturbance, dystarthria, interictal cerebellar deficit of extremely variable severity, and cerebellar atrophy; epilepsy has occasionally been described [9,10]. A dominant negative effect of the Ca\textsubscript{V2.1} mutated protein affecting the wild type product, rather than a haploinsufficiency, has been hypothesised [6,11–14].

Loss of function mutations of the mouse orthologue of the \textit{CACNA1A} gene cause recessive neurological phenotypes, in toettering (\textit{cacna1a}\textsuperscript{tg}; \textit{tg}), \textit{leaner} (\textit{cacna1a}\textsuperscript{tg-la}; \textit{tg-la}), \textit{rocker} (\textit{cacna1a}\textsuperscript{kr}) and rolling Nagoya (\textit{cacna1a}\textsuperscript{kr-rol}) mice [15,16]. In addition, one dominant \textit{cacna1a} mutation was recently described in the \textit{wobbly} mouse [17]. Homozygous mice for \textit{leaner}, \textit{tottering} and \textit{rocker} mutations are all affected by a more or less severe form of ataxia and absence epilepsy. \textit{Leaner} mice show the most severe ataxic phenotype with extensive degeneration of Purkinje cells and cerebellar granules. \textit{Tottering}, \textit{rocker}, \textit{rolling Nagoya} and \textit{wobbly} phenotypes are all caused by point mutations resulting in an amino acid exchange (P601L, T1310K, R1262G and R1255L, respectively), while \textit{leaner} is due to a mutation causing an aberrant splice and a premature translation termination codon (PTC), thus producing a truncated protein.

Besides the protein truncation, the PTC makes the mutated mRNA a good candidate for the quality control mechanism of nonsense-mediated decay (NMD), that selectively degrades mRNA harbouring PTC [reviewed in 18,19]. Should this be the case, a reduced expression of the mutant gene would be expected in \textit{leaner} mice.

A solid support to this hypothesis comes from mice carrying null or knockout \textit{cacna1a} mutations. Their phenotype overlaps that of \textit{leaner} mice, suggesting a strongly reduced or complete absence of the gene product in these mice as well [20,21]. Moreover, mice with knockdown mutations develop ataxia and cerebellar atrophy, with a severity of symptoms and a rate of progression inversely correlated with the amount of expressed Ca\textsubscript{V2.1} [22].

The hypothesis that \textit{cacna1a} mutations leading to PTC are subject to NMD also has implications for human EA2 mutations of the same kind, accounting for more than 60% of the total [10]. If mRNA turns out
to be degraded, no truncated protein will be present, thus indicating a haploinsufficiency mechanism rather than a dominant negative effect.

We have analysed the expression of cacna1a mRNA in brains of wild type and heterozygous and homozygous leaner pre-weaning mice. The results show a dramatic decrease of the cacna1aΔ6-b allele expression, compared to the wild-type allele, in the heterozygous and in the homozygous mutant mice, as well as a variable expression of mutant and wild-type cacna1a related to age.

2. Materials and methods

2.1. Animals

Experiments were performed on pre-weaning and adult mice. The C57BL/6-congenic leaner mutant strain with an oligosyndactylism marker gene (Os +/- cacna1aΔ6-b) was obtained from Jackson Laboratory (USA). Leaner homozygous mice were obtained by intercrossing Os +/- cacna1aΔ6-b. Offspring carrying 2 Os alleles (Os +/- Os +) die in utero [23]; those genotypically Os +/- cacna1aΔ6-b offspring have fused digits due to the Os allele; therefore, surviving offspring with normal digits were identified as leaner homozygous (+/cacna1aΔ6-b/cacna1aΔ6-b). In addition the genotypes of all mice here studied were confirmed by sequence analysis of genomic DNA.

2.2. cDNA amplification

Total brain RNA was isolated from homozygous and heterozygous leaner mice and wild type mice using Trizol® Reagent (Invitrogen) following the manufacturer’s protocols. After isolation, RNA preparations were digested with DNasel. cDNAs were synthesised from 4 μg of total RNA in 25 μl of volume using random hexamers with GeneAmp RNA PCR Kit Applied Biosystems N808-0143.

2.3. Semi-quantitative PCR

Two μl of the reverse transcription mix was amplified by PCR in the presence of “P”-dCTP using the following sets of primers: 1) 5′-AGG AAG AGA GGC ATC CTG ACC-3′ and 5′-CTC ATT GCC GAT AGT GAT GAC C-3′ for β-actin, used as internal control; 2) 5′-ACA GCC TTA TCG TCA CCA AC-3′ and 5′-ATG AAG CAC ATG CAT TAG GG-3′ for fragment 3140–3386 (Acc. no. MMU76716) of cacna1a, unmodified in the mutant; and 3) 5′-AGG AAG GGC GCA TGA AGG AG-3′ and 5′-GAG GTA GTG TCT GTC GTC TG-3′ for fragment 5771–5958 (Acc. no. MMU76716) of cacna1a, including the 98 nttron not spliced in the mutant form.

The PCR program was: 1 min at 94 °C, 1 min at 60 °C (first 5 cycles) or 58 °C (additional cycles) and 1 min at 72 °C for a total of 15 and 17 cycles. Preliminary experiments were done to determine the optimal number of cycles to ensure that the degree of amplification had a linear dependence on RNA concentration. Duplicate samples were analyzed.

Separation of the PCR products was performed on 6% polyacrylamide gel (PAGE), vacuum dried for 30 min at 80 °C, exposed to phosphor screen overnight and acquired by PhosphorImager ™ technology in a Storm 820 (GE Healthcare). Densitometric analysis was done using the ImageQuant (Molecular Dynamics, Sunnyvale, CA) software. The cacna1a mRNA expression was normalised over β-actin mRNA expression.

2.4. Quantitative Real-Time PCR (qRT-PCR)

PCR amplification and detection were performed using ABI Prism 7000 Sequence Detection System (Applied Biosystems) in a final volume of 25 μl. All PCR reactions (three reactions for each sample) were replicated at least four times and contained 100 ng of cDNA, 12.5 μl of TaqMan Universal Master Mix (Applied Biosystems) including the passive reference ROX, 1.25 μl of Assay on Demand Gene Expression Assay Mix for wild-type and mutated sequences (Applied Biosystems). The sequences of the PCR primers and probes (FAM dye labelled) were: 1) Forward: 5′-CCTAACAGGCACCCCAACTC-3′, Reverse: 5′-GGAGG-TAGTTGTCGCTTGTCGTCA-3′ and Probe: 5′-CAGTCCTGTGGAGATGCGGA-3′; 2) Forward: 5′-CGCGACGCTGTGGTGG-3′, Reverse: 5′-GAGTCTGAGTGTCTGATGGAGA-3′; and Probe: 5′-TCAGGCAGAGGG-3′ for mutation assay.

Negative controls were also assayed. The PCR reaction was performed under the following conditions: 50 °C for 2 min, 95 °C for 10 min and 40 cycles (95 °C for 15 s and 60 °C for 1 min). GAPDH amplification was used to normalise the reactions (TaqMan Control Reagents Kit Applied Biosystems).

As the efficiencies of the endogenous control and target PCR reactions were equivalent, the relative RNA abundance (RQ) was calculated according to the “comparative Ct method (ΔΔCt Method)” (available from http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_042380.pdf), where the amount of target, normalized to an endogenous reference and relative to a calibrator, is given by: 2−ΔΔCt. The relative cumulative expression (Relative Expression) of cacna1a and/or cacna1aΔ6-b alleles for each homozygous or heterozygous mouse was calculated as the ratio of the summed 2−ΔΔCt values of cacna1aWT and cacna1aΔ6-b (LN) mRNAs and the minimal value of the sums (calibrator) observed across the entire series.

3. Results

The relative expression levels of cacna1a in whole brain were determined initially by semi-quantitative analysis. Fig. 1 shows representative PAGE of wild type, heterozygous and leaner mice semi quantitative radioactive PCRs used to measure the mRNA levels of the wild type and mutant cacna1a genes. In this case, β-actin was used as internal control and the relative expression was revealed by densitometry of the 245 bp band of fragment 3140–3386 present in

![Fig. 1. Semiquantitative PCR analysis of normal (+) and leaner (tg-la) cacna1a alleles in wild type (P15 +/+), heterozygous (P11 and P16 +/tg-la) and leaner (P14 tg-la/tg-la) mice. Four PCR reactions are shown for each mouse: 1, β-actin; 2, fragment 3140–3386 unvaried in normal and mutant allele, here named cacna1a; 3, fragment 5771–5958 producing a 188 bp long band in normal allele and 285 bp if the intron has not been spliced; and 4, multiplex with all 3 pairs of primers. Note that a minimal amount of the aberrant splicing product is present also in wild type samples, this producing even an overestimate of the mutant allele in heterozygotes.](image-url)
The relative expression levels of \textit{cacna1a} mRNA in whole brain were then determined using the TaqMan qRT-PCR method, and were normalised against GAPDH RNA as endogenous control. The results are presented as Relative Expression (see Materials and methods) of homozygous and heterozygous mice (Fig. 2A) or Relative Quantity (RQ) calculated by 2\(^{(-\Delta \Delta C(T))}\) method [24] of \textit{cacna1a} \textsuperscript{+} vs \textit{cacna1a} \textsuperscript{tg-la} in heterozygous mice (Fig. 2B). Each sample has been analysed at least four times and data reported in the graphics are expressed as the mean ± 2×SEM.

Fig. 2A shows the Relative Expression of total \textit{cacna1a} mRNA of the three genotypes, wild type, heterozygote and leaner as a function of age. The total amount of mRNA in heterozygous mice has been obtained by summing the mRNA produced by \textit{cacna1a} \textsuperscript{+} and \textit{cacna1a} \textsuperscript{tg-la} alleles. Irrespective of age, the Relative Expression data of the three groups, wild types, heterozygotes and leaner mice, have been compared by the non-parametric Kruskal–Wallis 1-way ANOVA test. Their difference turned out to be highly significant (chi-square, 2 df = 12.21; P = 0.0022) (mean rank 14.29, 9.00 and 3.40 respectively). This was consistent with a decreased expression of the \textit{cacna1a} \textsuperscript{tg-la} allele. In addition, the expression increases with age in all the three genotypes, but the slope are much higher than in the other homozygous genotype (y-intercept: 9.14). Linear regression analysis in heterozygotes has not been applied because of their heterogeneous age. Fig. 2B shows the distribution of the RQs of \textit{cacna1a} \textsuperscript{+} and \textit{cacna1a} \textsuperscript{tg-la} alleles in heterozygous mice. Pre-weaning mice (P11 and P16) and 12 month old mouse show significantly lower expression of the \textit{cacna1a} \textsuperscript{tg-la} allele as compared to the normal one (Student’s t-test; P = 0.0005, P = 0.004 and P = 0.041, respectively). On the other hand, in elder mice (21 and 22 months old), this difference is not maintained. However, it must be noted that these mice have a total amount of RNA which is about half of that of wild type animals (Fig. 2A), as expected in diploid genotypes expressing only one gene.

4. Discussion
Leiner mice are affected by a severe form of recessive ataxia with symptoms as early as 8–10 days of age. Most of these mice die by weaning. The disease is due to a splice mutation of the \textit{cacna1a} gene, leading to a PTC and making the mRNA a possible candidate for degradation through NMD. Our results provide evidence of a very low level of mutant \textit{cacna1a} \textsuperscript{tg-la} mRNA in the brain of pre-weaning leiner mice, suggesting that NMD control on this gene is active at the onset of the disease. In addition, the relative quantity of the mutant allele increases with age, as shown in heterozygous animals, reaching almost the same level of the wild type allele during the second year of life. In this animals, however, the total amount of mRNA is about half of that of homozygous wild type animals. Different hypotheses could be proposed to explain this phenomenon. First of all, it could be the result of a decrement of NMD mechanism efficiency with neuronal development [25] and possibly with aging, as already observed in other cellular components of protein quality control such as ubiquitin-proteasome system or autophagic pathways [26,27]. On the other hand, we cannot exclude that the mutant mRNA and/or protein are more stable and the mRNA portion escaping NMD undergoes accumulation [28]. In any case, NMD decline is observed well after the disease onset.

The age dependent trend in the expression of \textit{cacna1a} \textsuperscript{tg-la} could also explain the conflicting results of previous measurements of \textit{cacna1a} \textsuperscript{tg-la} mRNA and protein by immunocytochemistry, in situ hybridization and northern-blot analysis. In fact, no difference between leiner and wild type mice was reported in some studies [29], while in others a very marked decrease or even the complete lack of mutant mRNA was observed [30].

All these data were obtained in mice showing an ataxic phenotype firstly described as recessive, while in humans mutations at the orthologous gene produce an autosomal dominant disorder. However, heterozygotes for both leiner and knockout mutations were recently shown to have a clear deficit in motor learning and in the vestibulo-ocular reflex [31], thus revealing a dominant effect of these mutations. In addition, in heterozygous leiner mutant mice also a reduced Ca\textsubscript{2+}L-mediated acetylcholine release at their neuromuscular junctions [32] and age-dependent motor and cognitive deficits [33] have been observed. The disease progression comparison of EA2 and the leiner phenotype strengthens even more the similarity between human and murine diseases. Although no longitudinal studies have been published, at our knowledge, on the natural history of the disease, EA2 is usually described as a non progressive disorder in contrast with the allelic disease SCAt, characterized by a progressive ataxia [34]. In our experience EA2 clinical picture is extremely variable over time: either decreasing or increasing its severity, but mostly being stable with age. This makes the leiner mice with their non progressive disorder a good animal model for studying the molecular mechanism underlying dominant inheritance of EA2 in man.

The finding that a mechanism degrading PTC-containing mRNAs, such as NMD, might have an effect on the \textit{cacna1a} gene has implications for the pathogenic mechanism of human EA2 mutations of the same type. A dominant negative effect of EA2 mutant Ca\textsubscript{2+}L \(\alpha\)-subunit on normal protein has been demonstrated by double transfection methods for mutations producing PTCs as well [6,11–14]. However, the present results on leiner mice and the similar phenotype of \textit{cacna1a} null,
knockout and knockdown mice [20–22] would indicate that also a haploinsufficiency mechanism can cause ataxia.

For this reason, a haploinsufficiency mechanism could be supposed in EA2 patients carrying CACNA1A mutations leading to PTCs (that account for ~60% of the total mutations), especially with early stop codon like those recently described in the first protein domain [10]. These latter cases are particularly significant because the truncating mutations involve the protein N-terminus, where the dominant negative effect over the wild type protein was shown [35].

The quality control mechanism degrading Cav2.1 mRNA carrying a PTC, however, appears to be age dependent. It may be possible that a haploinsufficiency mechanism in youngsters might be gradually substituted by a dominant negative effect in adulthood.

In conclusion, since in humans, as in mice, beyond a dominant negative effect, a mechanism of haploinsufficiency causing ataxia can be supposed in those patients carrying protein mutations producing PTCs, it is necessary to clear up the pathophysiological mechanism for each specific mutation, or class of mutations. This is because dominant negative effect and haploinsufficiency mechanisms work in opposite directions. Therefore, in the first case an effective therapy should disable a toxic molecule while in the second it should increase the expression of that particular product.

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