# Motor and cognitive deficits in the heterozygous *leaner* mouse, a $Ca_v2.1$ voltage-gated $Ca^{2+}$ channel mutant

Isabel Alonso [a,c], Joana M. Marques [b], Nuno Sousa [d], Jorge Sequeiros [a,c], I. Anna S. Olsson [b], Isabel Silveira [a,*]

[a] *UnIGENe, IBMC (Instituto de Biologia Molecular e Celular), Universidade do Porto, Portugal*
[b] *Laboratory Animal Science Group, IBMC (Instituto de Biologia Molecular e Celular), Universidade do Porto, Portugal*
[c] *ICBAS, Universidade do Porto, Portugal*
[d] *Neuroscience Group, Life and Health Sciences Research Institute (ICVS), Universidade do Minho, Portugal*

Received 12 December 2006; received in revised form 26 March 2007; accepted 5 April 2007
Available online 21 May 2007

## Abstract

The *leaner* mutation in mice affects the $Ca_v2.1$ voltage-gated calcium channel $\alpha_{1A}$-subunit gene (*Cacna1a*), causing a reduction in calcium currents predominantly in Purkinje cells. This reduction in calcium currents causes severe progressive cerebellar ataxia, beginning around postnatal day 10, in homozygous *leaner* mice ($tg^{la}/tg^{la}$), while their heterozygous littermates ($tg^{la}/+$) present no obvious behavioral deficits. In humans, heterozygous mutations in the *Cacna1a* orthologous gene produce a broad range of neurological manifestations. To evaluate the phenotypic status of the $tg^{la}/+$ animals, we assessed motor performance and cognition, at different ages, in these mutant mice. We were able to observe age-dependent impairment in motor and cognitive tasks; balance and motor learning deficits were found in demanding tasks on the rotarod and on the hanging wire test, while spatial learning and memory impairment was observed in the Morris water maze. Progressive dysfunction in escape reflexes, indicative of neurological impairment, was also present in $tg^{la}/+$ animals. Although not presenting major motor alterations, $tg^{la}/+$ mice show age-dependent motor and cognitive deficits.
© 2007 Elsevier Inc. All rights reserved.

*Keywords:* Rotarod test; Water maze test; Natural mutant; Calcium currents; Memory; Learning

## 1. Introduction

The *leaner* mutant ($tg^{la}$) mouse was first described in the 60s, as a cerebellar mutant (Sidman et al., 1965). Homozygous animals ($tg^{la}/tg^{la}$) are severely ataxic due to cerebellar atrophy, resulting from gradual degeneration of granule, Purkinje, and Golgi cells (Frank et al., 2003; Herrup and Wilczynski, 1982; Lau et al., 2004). Molecular analysis has shown a mutation in the splice donor consensus site of the *Cacna1a* gene, encoding the highly conserved brain specific $Ca_v2.1$ voltage-gated calcium channel (VGCC) $\alpha_{1A}$-subunit, which is highly expressed in the cerebellum and hippocampus (Day et al., 1996; Volsen et al., 1995). This mutation results in the truncation of the normal open reading frame beyond repeat IV and the expression of two novel $\alpha_1$ subunits with truncated carboxyl tails (Doyle et al., 1997; Fletcher et al., 1996). Although the homozygous mutants are extremely affected, their heterozygous littermates ($tg^{la}/+$) present no evident phenotypic abnormalities. Thus, the neurologically abnormal phenotype has been considered by several authors as inherited in an autosomal recessive manner (Fletcher et al., 1996; Meier and MacPike, 1971; Tsuji and Meier, 1971). Functional studies showed a reduction of approximately 30% in $Ca^{2+}$ conductance in $tg^{la}/+$ Purkinje cells. Channels harboring the *leaner* mutation in homozygosity showed a greater reduction in the calcium currents (Lorenzon et al., 1998).

* Corresponding author at: UnIGENe, IBMC, Rua do Campo Alegre, 823, 4150-180 Porto, Portugal. Tel.: +351 22 6074941; fax: +351 22 6099157.
*E-mail address:* isilveir@ibmc.up.pt (I. Silveira).

Reports on the expression of $\alpha_{1A}$-subunit levels are conflicting, with some studies reporting a reduction on *Cacna1a* transcript levels (Doyle et al., 1997), while others failed to observe differences in mRNA or protein expression amounts (Lau et al., 1998). This mutant has a diminished $Ca^{2+}$ buffering ability, attributed to reduced uptake by the endoplasmic reticulum and decreased expression of $Ca^{2+}$-binding proteins (Dove et al., 2000).

In humans, mutations in the *Cacna1a* orthologous gene (*CACNA1A*) have been implicated in three dominantly inherited disorders, with overlapping clinical features: the progressive spinocerebellar ataxia type 6 (SCA6), familial hemiplegic migraine type 1 (FHM1) and episodic ataxia type 2 (EA2) (Ophoff et al., 1996; Zhuchenko et al., 1997). At the mutational level, missense, nonsense and splice-site mutations, as well as a small $(CAG)_n$ expansion, are involved in these disorders, and the same molecular alteration may be associated with more than one phenotype in a single family (Alonso et al., 2003).

Calcium is a highly versatile and ubiquitous intracellular messenger, responsible for controlling, directly or indirectly, diverse cellular processes, including cell differentiation and proliferation, neurotransmitter release, transcription factor activation, apoptosis and synaptic plasticity (Berridge et al., 2000). Calcium plays a key role in the induction of activity-dependent synaptic plasticity in various central synapses, as a result of incoming information (Fitzjohn and Collingridge, 2002).

Modeling neurodegenerative disorders has provided important insights into protein function and disease pathogenic mechanisms. Animal model studies have also shown that usually mutation overexpression is required to produce detectable phenotypic alterations during the mouse lifespan (Watase and Zoghbi, 2003). Furthermore, the high diversity of clinical presentations shown in humans carrying the same *CACNA1A* mutation (Alonso et al., 2003), and the role of calcium homeostasis in normal brain function, have raised the hypothesis of subtle phenotypic alterations in $tg^{la}$/+ mice. To address this, we applied a battery of behavioral tests to assess motor and cognitive functions of $tg^{la}$/+ mice, throughout aging.

## 2. Methods

### 2.1. Animal husbandry

All the procedures described were approved by the local Ethics Committee and the Portuguese Chief Veterinary Office (Direcção Geral de Veterinária).

Breeder pairs of heterozygous $tg^{la}$/+ mice were acquired from The Jackson Laboratory (Bar Harbor, Maine, USA) and

Table 1
Number of animals used in each protocol

| Behavioral test | Age (months) | wt | | $tg^{la}$/+ | |
| --- | --- | --- | --- | --- | --- |
| | | Male | Female | Male | Female |
| Rotarod first protocol | 6 | 5 | 5 | 5 | 5 |
| | 12 | 6 | 5 | 4 | 4 |
| | 22 | 5 | 5 | 5 | 5 |
| Rotarod second protocol | 6 | 10 | 10 | 10 | 10 |
| | 12 | 10 | 10 | 9 | 10 |
| | 22 | 9 | 7 | 8 | 6 |
| Water maze—acquisition | 6 | 10 | 10 | 10 | 10 |
| | 12 | 10 | 10 | 9 | 10 |
| | 22 | 9 | 7 | 8 | 6 |
| Water maze—distractor cue | 6 | 10 | 10 | 10 | 10 |
| | 12 | 10 | 10 | 9 | 10 |
| Water maze—cued | 6 | 10 | 10 | 10 | 10 |
| | 12 | 10 | 10 | 9 | 10 |
| Hanging wire | 6 | 5 | 5 | 5 | 5 |
| | 12 | 5 | 5 | 7 | 5 |
| | 22 | 4 | 2 | 4 | 2 |
| Clasping behavior | 6 | 5 | 5 | 5 | 5 |
| | 12 | 5 | 7 | 8 | 8 |
| | 22 | 4 | 2 | 4 | 2 |

were housed in the IBMC animal facility. Mice were bred to produce heterozygous $tg^{la}$/+ offspring. Heterozygous $tg^{la}$/+ breeders are unable to generate wild-type offspring, as the $tg^{la}$ alteration is inherited in repulsion with the oligosyndactylism mutation, which is embryonically lethal in homozygosity. *Leaner* heterozygous mice are, thus, heterozygous for olygosyndactylism, which causes a skeletal and renal phenotype, resulting in fusion of the second and third digits of all four paws and reduction of nephrons in the kidney (Wise and Pravtcheva, 2004). As no neurological phenotype has been described associated to olygosyndactylism, interference with the motor and cognitive functions assessed in this study is not expected. An additional colony of animals with genotype C57BL/6J:+/+ was maintained, as controls. Mice were housed and bred in a constant-temperature room, with food and water given *ad libitum* and a 12 h light–dark cycle. Mice of both genders, heterozygous and wild-type aged 6, 12 and 22 months were studied. The number, age and gender of animals tested in each protocol are described in Table 1.

## 2.2. Genotyping

*Leaner* heterozygous mice can easily be distinguished by the presence of fusion of the second and third digits of all four paws. Nevertheless, genotype was confirmed by restriction enzyme analysis after PCR amplification. After euthanasia, the mouse tail tip was cut and DNA was extracted after lysis at 55 °C in buffer (400 mM NaCl, 10 mM Tris–HCl pH 7.5, 2 mM EDTA pH 7.5, 1% SDS and 0.2 mg proteinase K), overnight. Following incubation, 2 mM NaCl (final concentration) was added to the lysate, and the samples were mixed and centrifuged at 16,000 rpm. DNA was, then, precipitated with ethanol and centrifuged at 16,000 rpm. The DNA pellet was resuspended in water. PCR amplification was performed using the previously described primers TGLA-1 (acgaaggcggcatgaaggaga) and TGLA-5R (ttccatggggaggtagtgtt) (Wakamori et al., 1998). PCR products were digested with *Bse*DI (Fermentas, Ontario, Canada). The digested products were submitted to electrophoresis on a 2.0% agarose/Et-Br gel, yielding the following fragments: 7, 28, 99 and 161 bp, in wild-type, and 7, 28, 99, 161, and 260 bp, in $tg^{la}$/+.

## 2.3. Motor performance test

Rotarod tests were carried out to assess coordination and balance functions. Mice were tested using an accelerated rotarod (TSE systems, Bad Homburg, Germany), according to two different protocols. The first consisted of four trials per day (10 min rest between trials), on four consecutive days. During a trial, the rod accelerated from 4 to 40 rpm, over 5 min, and then remained at 40 rpm for an additional 5 min. Each trial lasted until the mouse fell from the rod, or for a maximum of 10 min. The results were averaged across the four daily sessions. This first protocol was applied to a

pilot group of animals, and data analysis suggested motor learning impairment. To confirm this hypothesis a second, more demanding, rotarod protocol was applied to a different group of animals. In this protocol, three sessions of four trials each (1 h rest between sessions), on seven consecutive days were applied to mice aged 6 and 12 months. Data were averaged over the three daily sessions. As we expected reduced physical endurance in 22-month-old mice, they were tested in a 7-day protocol with only one daily session, and results are presented averaged over the four daily trials. This second group of animals continued behavioral testing, with water maze, hanging wire and clasping behavior assessment. For each age tested, a different group of animals was studied.

## 2.4. Hanging wire test

In this test, the animals were placed, by the forelimbs, on a stainless steel bar (30 cm length, 2 mm in diameter, and elevated 30 cm from the surface), at a point midway between the supports, and observed for 60 s. The number of seconds spent hanging was recorded, in three consecutive trials per animal, with a 30 s interval between trials.

## 2.5. Clasping behavior

Clasping was measured in a tail suspension test, in which the mouse was held by the tail at a 30 cm height from the bench surface, and observed for a period of 30 s. Wild-type mice will tend to hold their legs away from the main trunk, with the toes, at least on the hind feet, splayed.

## 2.6. Morris water maze

The cognitive status of the animals as regards spatial learning and memory was assessed with the Morris water maze. Briefly, the maze consisted of a grey circular polypropylene tank, 1 m in diameter and 0.5 m deep, which was filled with water, made opaque through the addition of powdered milk, to a 35 cm depth. The maze was divided in four imaginary equal-sized quadrants, and an escape platform (12 cm × 9 cm) was placed in the pool. The swim path was recorded by the computerized VideoMot 2 tracking system (TSE systems, Bad Homburg, Germany). The maze was located in a room containing extramaze visual cues. The Morris water maze protocol consisted of a 2-day training period in the cued, fixed platform position procedure, followed by the hidden platform paradigm with a probe, and an additional probe performed 7 days after the acquisition. Mice aged 22 months were not submitted to behavioral testing after this second probe as we did not expect that the aged mice of both genotypes were able to cope with a more extended protocol. Mice aged 6 and 12 months continued the water maze protocol with cued learning in the presence of a distractor cue and finally a cued, variable platform position procedure.

### 2.6.1. Spatial learning, fixed platform position acquisition procedure

Each mouse was given 28 trials to locate the hidden platform, in blocks of four trials a day, for seven consecutive days. Throughout the acquisition protocol the platform remained in the same position and the latency, pathlength and speed taken to reach the escape platform were recorded. Each trial had a maximum of 60 s. Between trials, mice were allowed to rest for 60 s on the platform. The results were averaged across three daily trials (excluding the first trial of each day). On the last day, a probe test was also performed, in which the platform was removed from the pool, and each animal was allowed to search for the platform for 60 s. The amount of time spent in each quadrant was recorded. Seven days later the probe was repeated to assess memory recall. For mice aged 22 months the acquisition protocol was adapted to 27 trials, in blocks of three trials a day for nine consecutive days. Data are presented as average across two daily trials (excluding the first trial of each day). On the 10th day, only the probe test was performed. Memory recall was assessed 7 days later.

### 2.6.2. Distractor cue procedure

Mice from each genotype received 4 days of training (four trials per day), in which the platform was made visible by a flag. Its position varied in each trial, and an additional cue, irrelevant to the platform position, was located in the quadrant opposite to the platform (distractor cue). A different distractor cue was used on each of the four daily trials, with a diverse location on each trial. The four distractor cues used were a black and white cube, a black and white sphere, a black and white cross or a black and white triangle. Similarly to the previous tests, the latency, pathlength and speed to reach the platform were recorded. Results were averaged over three daily trials (excluding the first trial of each day).

### 2.6.3. Cued learning, variable platform position procedure

The test was performed in a single day, the platform was made visible by a flag and its position changed between each of the eight trials. No additional intramaze cues were applied, and escape latency, pathlength and speed were recorded. Data are presented averaged across the seven trials (excluding the first trial).

### 2.7. Statistical analysis

Statistical analysis was performed using SPSS (version 14 for Windows). The effect of genotype and gender on motor performance in the rotarod and on the cognitive capacity, in the Morris water maze acquisition and distractor cue tests, was examined by repeated-measures ANOVA. For the body weights, the effect of genotype and gender was assessed by two-way univariate analysis of variance (ANOVA). For the hanging wire, probe trials and cued learning, comparisons between groups were performed using Student's unpaired *t*-test. For the probe trials a Student's *t*-test for difference from

chance was also applied to assess learning of the platform location, within each genotype. In case of categorical variables, as the clasping behavior quantifications, a $\chi^2$ test was used. Differences were considered significant if $p < 0.05$.

## 3. Results

### 3.1. Normal body weight

The average body weight (g) increased with age for both genotypes and was $24.90 \pm 1.51$ for controls and $24.67 \pm 1.39$ for $tg^{la}/+$, at 6 months; $30.02 \pm 1.08$ for controls and $29.05 \pm 0.93$ for $tg^{la}/+$, at 12 months and $35.13 \pm 0.91$ for controls and $31.67 \pm 1.90$ for $tg^{la}/+$, at 22 months. Males were heavier than females at all ages ($p < 0.005$ at 6, 12 and 22 months). When compared to controls, $tg^{la}/+$ showed similar body weights both at 6 months ($p = 0.82$) and 12 months ($p = 0.58$). At 22 months, differences were found between genotypes due to a reduced weight gain in $tg^{la}/+$ females ($p < 0.05$).

### 3.2. Age-dependent motor impairment on the rotarod

Motor coordination and balance was assessed on a rotarod apparatus. The ability to maintain balance was evaluated by the latency to fall from the accelerating rotarod, while the response to repeated training is a measure of motor learning. The performance on the rotarod was similar between $tg^{la}/+$ and wild-type animals, when the first and less demanding protocol was applied. Both genotypes improved their performance with training at 6 months [$F_{1.91,34.46} = 17.616$, $p < 0.005$], 12 months [$F_{3,51} = 14.715$, $p < 0.005$] and 22 months [$F_{1.76,31.61} = 30.873$, $p < 0.005$] (data not shown). When compared to controls, $tg^{la}/+$ mice showed similar performances, at 6 months ($p = 0.49$), 12 months ($p = 0.46$) and 22 months ($p = 0.94$) (data not shown). When the second and more challenging protocol was applied, differences between genotypes were observed (Fig. 1). At 6 months of age, no statistically significant differences were detected ($p = 0.32$), whereas at 12 months, $tg^{la}/+$ performed significantly worse than wild-type mice ($p < 0.05$) (Fig. 1). At 22 months, as both genotypes were thought to not stand this complete challenging protocol, only one daily session was applied and no statistically significant differences were found ($p = 0.28$). As in the first protocol, the amount of time spent on the accelerating rotarod increased over time, for both genotypes at 6 months [$F_{2.13,80.85} = 22.936$, $p < 0.005$], 12 months [$F_{2.81,103.92} = 35.237$, $p < 0.005$], and 22 months [$F_{3.11,90.10} = 15.812$, $p < 0.005$]. No interaction was observed between genotype and gender at any of the studied ages.

### 3.3. Motor and neurological dysfunction

In the hanging wire test, we observed differences between $tg^{la}/+$ and wild-type mice, mainly at 6 and 12 months of age.
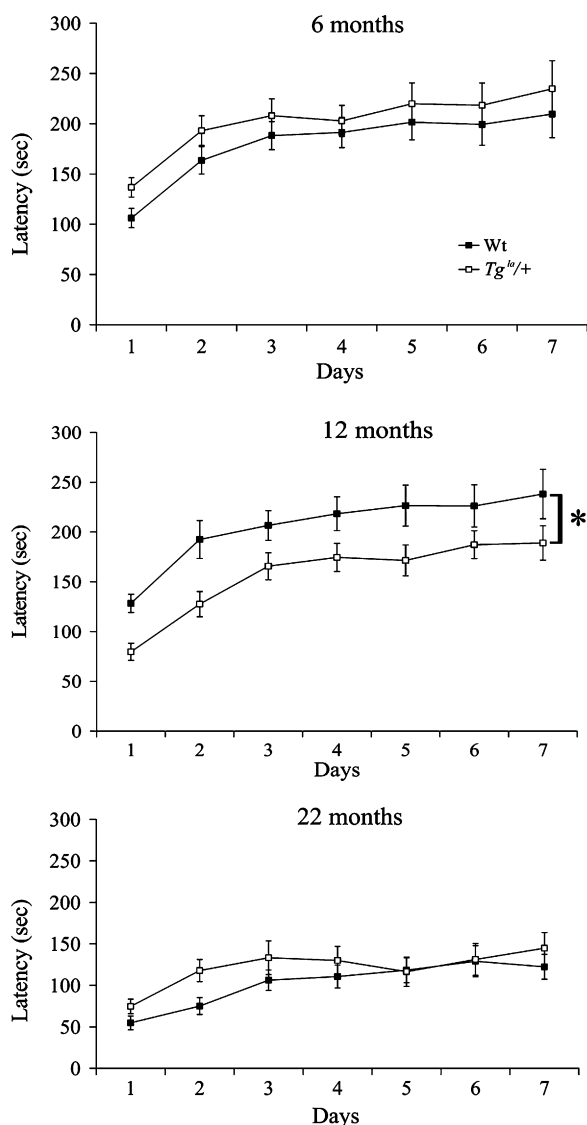
Fig. 1. Rotarod test for motor coordination and learning. Time (seconds) spent on the accelerating rod across 7 days, with three daily sessions, for $tg^{la}/+$ and wild-type mice, tested at 6 and 12 months of age. Data expressed as mean ± S.E.M. of three daily sessions, each comprising four trials in seven consecutive days. Both genotypes, aged 22 months, performed 7 days of testing with only one daily session. Data expressed as mean ± S.E.M. of one daily session comprising four trials in seven consecutive days. *$p < 0.05$ for control vs. $tg^{la}/+$ mice.
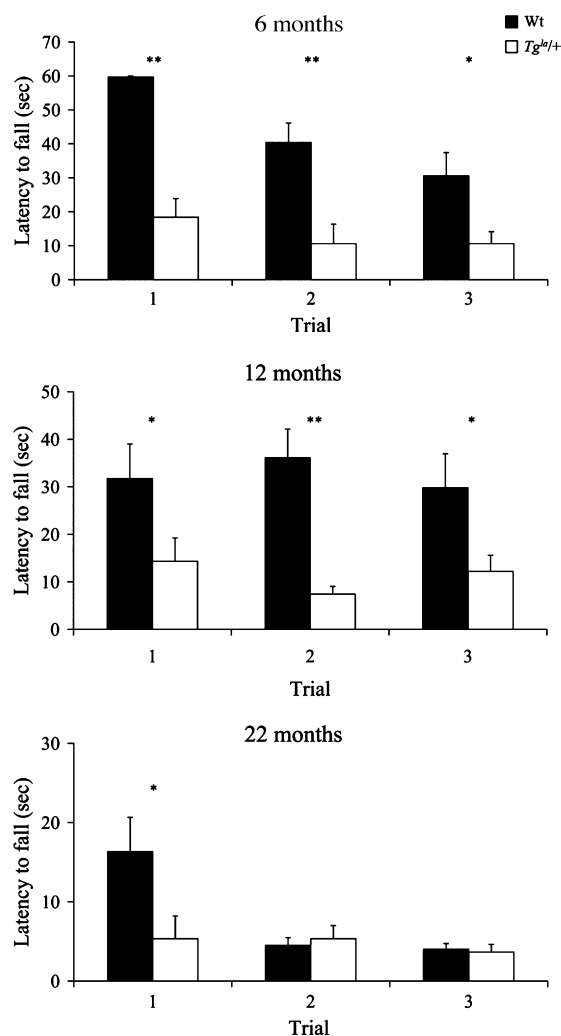


Fig. 2. Hanging wire test. Latency to fall from the hanging wire (seconds) across trials for $tg^{la}/+$ and wild-type mice. All mice performed only one session with three trials. Data expressed as mean ± S.E.M. of each trial. *$p < 0.05$ and **$p < 0.005$ for control vs. $tg^{la}/+$ mice.

mals it was only detected in 17 and 50% of the animals at 12 and 22 months. Differences between $tg^{la}/+$ and wild-type mice were statistically significant at all ages tested ($p < 0.005$) (Fig. 3).

### 3.4. Spatial learning impairment

#### 3.4.1. Acquisition and recall

Spatial learning performance of $tg^{la}/+$ mice proved to be different when compared to wild-type, with $tg^{la}/+$ mice showing increased pathlength and latency to reach the platform (Fig. 4 and S1). Comparisons between genotypes showed that $tg^{la}/+$ mice had, relative to wild-type, longer pathlength and latency, at 6 and 22 months ($p < 0.005$ for each test at 6 months, and $p < 0.05$ for each test at 22 months) (Fig. 4A and S1), whereas at 12 months, no differences were found between genotypes in pathlength ($p = 0.27$) or latency ($p = 0.35$). Swim speed comparisons showed no dif-

*Leaner* heterozygous mice spent significantly less time hanging from the wire in all trials, both at 6 months ($p < 0.005$ in first and second trials; $p < 0.05$ in third trial) and 12 months ($p < 0.05$ in first and third trials; $p < 0.005$ in second trial). For mice aged 22 months, significant differences between genotypes were only found in the first trial ($p < 0.05$), whereas in the second and third trials no differences between genotypes were found ($p = 0.95$, $p = 0.60$) (Fig. 2), as wild-type animals also performed poorly.

The clasping behavior was present in all the observed $tg^{la}/+$ mice, since age 6 months, whereas in wild-type ani-
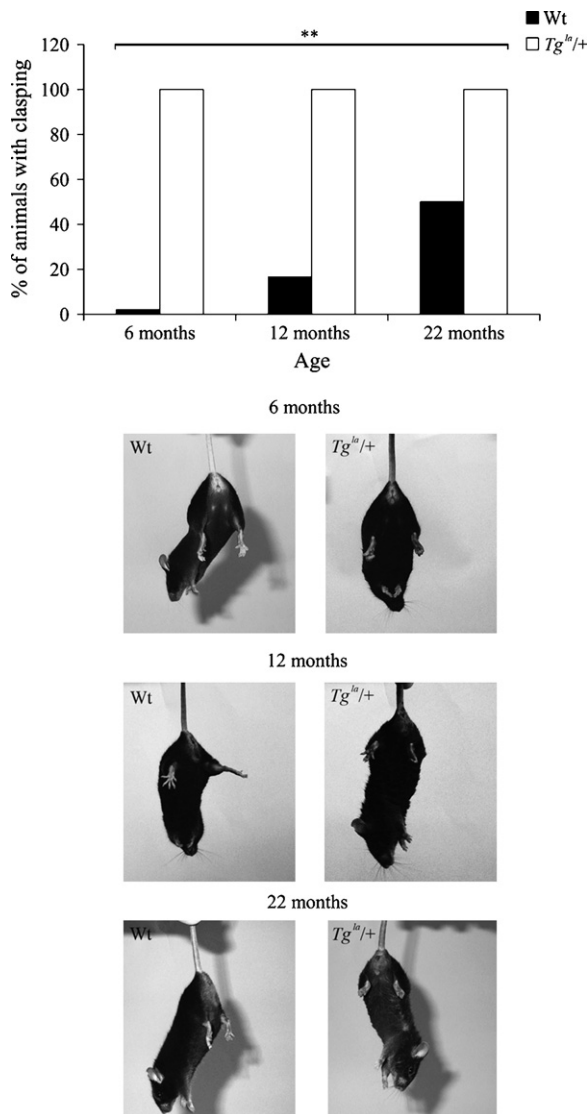
Fig. 3. Clasping behavior. The graphs show the percentage of animals presenting the clasping phenotype in the tail suspension test. Photographies of the clasping phenotype at age tested illustrate normal escape reflexes in controls, contrasting to the clasping phenotype shown by $tg^{la}/+$ mice. **$p < 0.005$ for control vs. $tg^{la}/+$ mice.

ferences between genotypes at 12 months ($p = 0.64$) and 22 months ($p = 0.31$), while at 6 months $tg^{la}/+$ mice presented swim speeds lower than controls ($p < 0.005$). In the acquisition phase, overall pathlength decreased over days, for both $tg^{la}/+$ and controls, at 6 months [$F_{4.40,154.12} = 14.887$, $p < 0.005$] and 12 months [$F_{3.20,112.13} = 9.907$, $p < 0.005$]. At 22 months pathlengths did not change significantly throughout trials [$F_{2.30,59.85} = 1.448$, $p = 0.24$], for any of the genotypes (Fig. 4A). The latency to find the escape platform diminished over trials, for both genotypes, at all ages, and no significant differences were found in swim speed over trials (see supplementary data S1). No interaction was observed between genotype and gender at any of the studied ages.

Mice of both genotypes learned the platform location, at all ages, showed by the finding that the percentage of time spent in the target quadrant was significantly greater than chance (25%) ($p < 0.005$ for both genotypes at 6 and 12 months; $p < 0.05$ for both genotypes at 22 months). Comparisons between genotypes showed that when analyzing the first 30 s of the probe trial, $tg^{la}/+$ mice spent less time in the target quadrant than wild-type, at all ages, although the difference was only significant at 6 months of age ($p < 0.05$) (Fig. 4B). A similar trend was observed when the whole probe trial (60 s) was analyzed; for the first 15 s, however, no differences were found between genotypes at any of the studied ages (data not shown). In the probe test performed 7 days after acquisition, to assess memory recall, animals of both genotypes remembered the location of the platform with performance being significantly better than chance, at 6 and 12-month-old ($p < 0.005$ for both genotypes at 6 months; $p < 0.005$ for control animals with 12 months and $p < 0.05$ for $tg^{la}/+$ mice with 12 months). In aged animals (22 months) only control animals showed a significant increase in the percentage of time spent in the target quadrant when compared to chance (25%) ($p < 0.05$), whereas for the $tg^{la}/+$ mice this difference was not observed ($p = 0.17$). Comparisons between genotypes showed that when analyzing the time spent in the target quadrant, no significant differences between genotypes were found, when the first 30 s were analyzed (at 6 months $p = 0.67$, 12 months $p = 0.85$ and 22 months $p = 0.44$) (Fig. 5). Similar results were obtained for the first 15 s and for the whole 60 s trial (data not shown).

### 3.4.2. Distractor cue test

In the water maze test with the presence of a distractor cue, at 6 months no differences were found between *leaner* heterozygous and control mice in pathlength ($p = 0.76$), latency ($p = 0.57$) or swim speed ($p = 0.24$) (see supplementary data S2). In mice aged 12 months no differences were observed between groups in pathlength ($p = 0.87$) or latency ($p = 0.20$), while lower swim speeds were observed in $tg^{la}/+$ mice when compared to controls ($p < 0.05$). In the presence of a distractor cue, both genotypes improved their performance with training, reducing the pathlength at 6 months [$F_{3,108} = 14.912$, $p < 0.005$] and 12 months [$F_{2.29,80.12} = 22.424$, $p < 0.005$] (see supplementary data S2). Escape latency also diminished with training for both studied ages, whereas in swim speed differences were only found at 12 months (see supplementary data S2). No interaction was observed between genotype and gender at any of the studied ages.

### 3.5. Increased pathlength in non-spatial tasks

In the cued task, applied to test non-spatial reference memory, pathlength comparisons between genotypes revealed no differences at 6 months ($p = 0.19$). Conversely, at 12 months $tg^{la}/+$ showed longer pathlength than controls ($p < 0.05$) (Fig. 6). Comparisons of latency and swim speed showed no differences between $tg^{la}/+$ and controls at 6 months
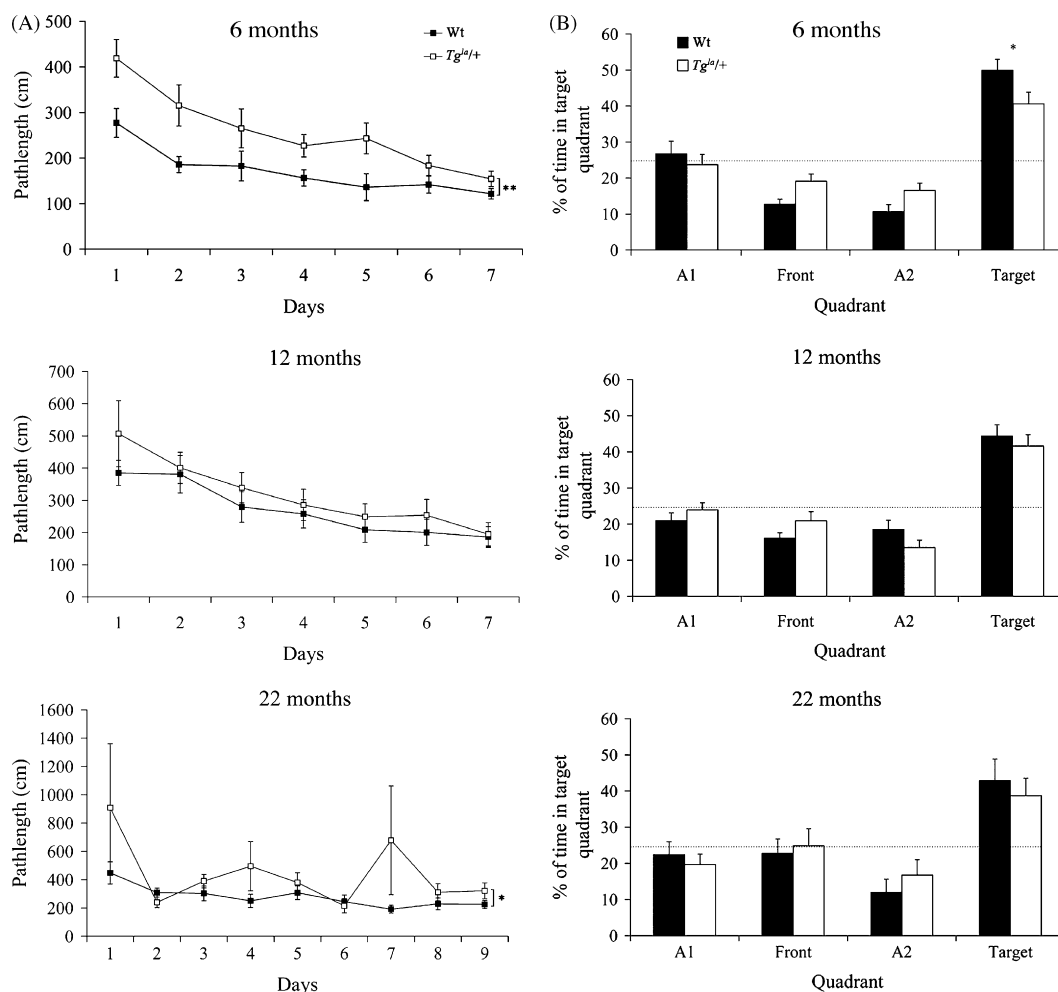
Fig. 4. Water maze test for spatial learning and memory. (A) Pathlength (cm) across days of training, for $tg^{la}/+$ and wild-type mice, in the hidden platform paradigm. Mice with 6 and 12 months performed one daily session with four trials, whereas mice with 22 months performed one daily session with three trials. Data expressed as mean ± S.E.M. of three daily trails (first trial of each day was excluded) for animals with 6 and 12-month-old, and two daily trials (first trial of each day was excluded) for mice with 22 months. (B) Percentage of time spent, in each of the four imaginary quadrants, for $tg^{la}/+$ and wild-type mice during the first 30 s of the probe trial. Dashed line represents chance performance (25%). Data expressed as mean ± S.E.M. *$p < 0.05$ for control vs. $tg^{la}/+$ mice.

($p = 0.95$, $p = 0.18$), or at 12 months ($p = 0.54$, $p = 0.10$) (see supplementary data S3).

## 4. Discussion

### 4.1. Neurobehavioral deficits in $tg^{la}/+$

The main purpose of the present study was to identify impaired neurobehavioral features, throughout aging, in a mouse with a heterozygous mutation in a calcium channel gene. We applied a battery of behavioral tests to characterize the influence of reduced calcium currents through $Ca_v2.1$ voltage-gated $Ca^{2+}$ channels in motor and learning capacities of $tg^{la}/+$ mice. We examined motor coordination, learning and memory over the lifespan of $tg^{la}/+$, compared to wild-type mice. Although $tg^{la}/+$ mice have been considered, by several authors, healthy and phenotypically identical to wild-type, the behavioral responses now observed were

remarkably different from the wild-type, evident at 6 months and aggravated with aging.

### 4.2. Motor coordination impairment

*Leaner* heterozygous mice do not show obvious impairment in motor coordination or balance, since their performance is not different from the wild-type in a standard rotarod protocol. However, when a more demanding rotarod protocol of three sessions, with four trials each, was applied, 12-month-old $tg^{la}/+$ animals showed motor deficits. In older animals (22 months) no significant genotype effect was found, however, these mice were submitted to an intermediate protocol, thus we cannot exclude that these aged animals have altered motor function in more demanding tests. Nevertheless, $tg^{la}/+$ mice present impaired performance in physically challenging rotarod protocols, suggestive of motor learning deficits. Recently, impaired motor learning in the vestibulo-ocular reflex has been observed in *leaner*
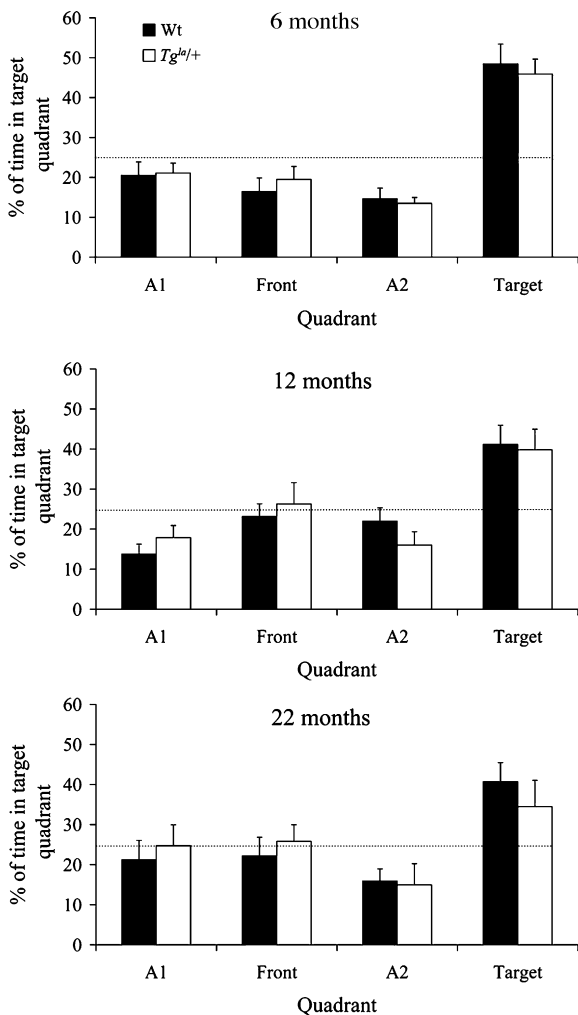
Fig. 5. Probe trial performed 7 days after the acquisition period to assess memory recall. Percentage of time spent in each of the four imaginary quadrants, for $tg^{la}$/+ and wild-type mice during the first 30 s of the probe recall trial. Dashed line represents chance performance (25%). Data expressed as mean ± S.E.M.
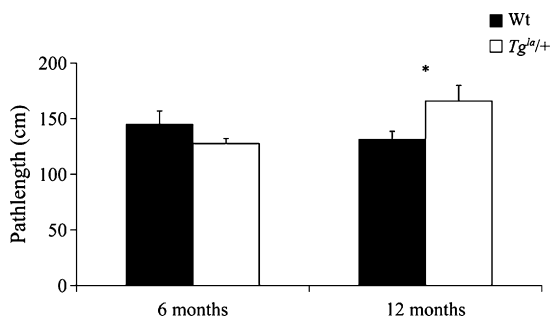


Fig. 6. Water maze cued learning, variable platform position procedure. Pathlength (cm) across trials, for $tg^{la}$/+ and wild-type mice, in the cued, variable platform position paradigm. Mice with 6 and 12 months performed eight trials in a single day. Data expressed as mean ± S.E.M. of seven trials (first trial was excluded). *$p < 0.05$ for control vs. $tg^{la}$/+ mice.

heterozygous and *Cacna1a* knockout mice, suggesting that minor alterations in $Ca_v 2.1$ currents can impair motor function (Katoh et al., 2007), and reinforcing the hypothesis of the presence of motor learning deficits in this mouse mutant. Although the rotarod motor function impairment in $tg^{la}$/+ mice was observed only in aged mice (12 months), motor dysfunction can be observed as early as at 6 months when the hanging wire task is applied. When performing this task, mice with strong and coordinated motor functions hold and climb onto the wire and escape. Conversely, mice with deficient motor skills show difficulty in holding onto the wire and fall more quickly. *Leaner* heterozygous mice presented early-onset (6 months) motor incoordination and/or reduced muscle strength on the hanging wire. Notably, performance in this task is affected by age in both genotypes: at 22 months genotype differences were only found in the first trial as wild-type animals were also not able to efficiently complete this task over trials. The motor impairment of the $tg^{la}$/+ mice in challenging rotarod protocols and in refined motor tasks, like the hanging wire, indicates that this mutant exhibit altered cerebellar function. Alterations in $Ca^{2+}$ influx through neuronal cells are likely to be the molecular basis of $tg^{la}$/+ age-dependent deficits in motor learning, in physically challenging protocols. The motor changes observed in $tg^{la}$/+ mice are similar to those displayed by null mouse mutants for calcium binding proteins (CaBPs). These mutants show impairment in motor performance when challenged, even without histopathological or evident phenotypic alterations, caused by deficits in calcium buffering and modulation of calcium channel activity (Airaksinen et al., 1997; Schwaller et al., 2002). *Leaner* heterozygous mice also showed early-onset (6 months) and progressive clasping behavior, a stereotyped phenotype indicative of neurological dysfunction of motor control pathways, which is present in animal models of several neurodegenerative disorders, such as Machado–Joseph disease (Cemal et al., 2002), Huntington's disease (Mangiarini et al., 1996) and spinal and bulbar muscular atrophy (McManamny et al., 2002) mouse models, as well as in a Rett syndrome mouse model (Guy et al., 2001), even though the basis for this behavioral alteration is currently unknown.

These results show that $tg^{la}$/+ mice have variable degrees of motor impairment, which is observed earlier (6 months) in more complex motor coordination tasks, and progresses with age. One can speculate that reduced calcium currents in $tg^{la}$/+ cerebellar neuronal cells, where $Ca_v 2.1$ calcium channels are highly expressed (Volsen et al., 1995), could induce dysfunction in calcium homeostasis and, consequently, alteration of spine morphology or deficient cerebellar maturation, resulting in motor impairment. Additionally, it has been reported that in *leaner* homozygous mice a neuromuscular junction (NMJ) functional phenotype is also present, characterized by a 50% reduction on acetylcholine release, which is compensated by non-$Ca_v 2.1$ channels (Kaja et al., 2007). Our study does not exclude the possibility that alterations in $tg^{la}$/+ NMJ contribute to the motor deficits reported here.

### 4.3. Spatial learning and memory deficits

The $tg^{la}$/+ mutant has age-dependent spatial learning and memory impairment in the Morris water maze, when compared to wild-type, shown by an increase in pathlength and time spent searching for the platform. Despite the motor dysfunction described above, $tg^{la}$/+ mice are able to perform the water maze task, though performances differ with age. Mice with 6 months, even though presenting spatial learning deficits, showed by increased pathlengths, are able to locate the hidden platform position. The spatial learning deficits in $tg^{la}$/+ mice are corroborated by a reduction, relative to controls, on time spent in the target quadrant observed in the probe trial. At 12 months, although showing more difficulties in learning the location of the hidden platform, $tg^{la}$/+ mice were able to learn the task at levels of performance comparable to wild-type, confirmed by the similar performance in the probe trial. The results obtained in mice aged 12 months may suggest that additional features are contributing to the reduced performance in younger $tg^{la}$/+ mice. At 6 months, we can hypothesize that a deficit in attention (though no differences were observed in performance between genotypes in the presence of a distractor cue) or motivation (as only $tg^{la}$/+ mice aged 6 months swim at a significantly slower speed when compared to wild-type) might explain the apparently cognitive findings. However, in aged mutant animals (22 months) water maze performance was significantly different from controls, suggestive of spatial learning deficits, though both genotypes had similar performances in the probe trial. At 22 months, as no differences were observed in swim speed or in the visible fixed platform paradigm, performed before place learning, visual or motor impairment do not seem to be contributing to the learning deficits observed. Nevertheless, we cannot rule out some influence of background on variability which can mask larger differences in motor and learning behavior as we were not able to use littermate control animals. In aged (22 months) $tg^{la}$/+ animals we also observed a memory deficit showed in the probe recalling, in which the time spent in the target quadrant did not differ from chance.

Calcium influx into the postsynaptic neuron through *N*-methyl-D-aspartate (NMDA) receptors and VGCC, induces long-term potentiation (LTP), a type of synaptic strengthening responsible for behavioral changes exhibited with the learning process (Bliss and Collingridge, 1993; Dunwiddie and Lynch, 1979; Grover and Teyler, 1990). This $Ca^{2+}$ influx induces transcription of activity-dependent genes and *de novo* protein expression, essential for the establishment of LTP, and thus for the acquisition of memory. Spatial learning and LTP dysfunction have been reported in mutants for several $Ca^{2+}$ regulated proteins (Abeliovich et al., 1993a,b; Bourtchuladze et al., 1994; Kang et al., 2001), suggesting that $Ca^{2+}$ signaling transduction is essential for LTP and for the adoption of a spatial orientation strategy. The high expression of P/Q-type calcium channel $\alpha_{1A}$-subunit in dentate gyrus granule cell and molecular layers, as well as in

pyramidal cells from the CA3 region (Day et al., 1996), suggests that reduced $Ca^{2+}$ influx through these neurons in $tg^{la}$/+ mice may result in hippocampal dysfunction and LTP impairment, which can be responsible for the spatial learning deficits reported here. The disruption of $\alpha_1$-subunit interaction with $Ca^{2+}$/calmodulin in *leaner*, the latter complex known to contribute to calcium-dependent synaptic plasticity (Lee et al., 1999), could alternatively be the basis for the learning and memory impairment observed in the $tg^{la}$/+ mutant mice.

### 4.4. Deficits in the heterozygous leaner mouse and human neurological disorders

Several types of human spinocerebellar ataxias, which are mainly characterized by progressive late-onset gait and limb ataxia, also show cognitive impairment (Manto, 2005). On the other hand, *CACNA1A* mutations in humans cause a broad range of symptoms, including progressive or episodic ataxia and hemiplegic migraine, which may occur isolated or simultaneously, in the same patient or family. As expected according to the cognitive impairment, ranging from marked learning difficulties to major mental retardation, shown by patients with *CACNA1A* mutations (Denier et al., 1999; Kors et al., 2003; Ophoff et al., 1996), we observed learning deficits in the $tg^{la}$/+ mice. This suggests that molecular and electrophysiological mechanisms underlying the cognitive deficits in $tg^{la}$/+ can provide insight into the pathophysiology of cognitive diseases. The $tg^{la}$/+ mutant mouse can, thus, be used as a model to study some of the aspects of cognitive diseases. Moreover, these results show that in mice, as observed in humans, the presence of a heterozygous mutation in the *Cacna1a* gene is sufficient to produce a disease phenotype during the mouse lifespan, resulting in a wide range of deficits. Additionally, these data put forward the hypothesis that like in the $tg^{la}$/+, the other known *Cacna1a* heterozygous natural mutants, namely *tottering* (Fletcher et al., 1996), *rolling Nagoya* (Mori et al., 2000) and *rocker* (Zwingman et al., 2001), may also present minor motor and cognitive deficits. Furthermore, mouse models of autosomal dominant neurological disorders usually require the overexpression of the mutant protein to show a disease phenotype during their short lifetime.

In summary, our results show that a heterozygous mutation in the $Ca_v2.1$ voltage-gated $Ca^{2+}$ channel $\alpha_{1A}$-subunit in the *leaner* mouse causes age-dependent cognitive impairment and motor incoordination in refined tasks, as well as age-related and progressive alterations in escape reflexes. These gene-associated deficits demonstrate that $Ca_v2.1$ $Ca^{2+}$ channels are involved, not only in motor activity, but also in learning and memory.

### Disclosure statement

There are no conflicts of interest to disclose.

## Acknowledgments

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neurobiolaging.2007.04.005.

## References

Abeliovich, A., Chen, C., Goda, Y., Silva, A.J., Stevens, C.F., Tonegawa, S., 1993a. Modified hippocampal long-term potentiation in PKC gamma-mutant mice. Cell 75, 1253–1262.

Abeliovich, A., Paylor, R., Chen, C., Kim, J.J., Wehner, J.M., Tonegawa, S., 1993b. PKC gamma mutant mice exhibit mild deficits in spatial and contextual learning. Cell 75, 1263–1271.

Airaksinen, M.S., Eilers, J., Garaschuk, O., Thoenen, H., Konnerth, A., Meyer, M., 1997. Ataxia and altered dendritic calcium signaling in mice carrying a targeted null mutation of the calbindin D28k gene. Proc. Natl. Acad. Sci. U.S.A. 94, 1488–1493.

Alonso, I., Barros, J., Tuna, A., Coelho, J., Sequeiros, J., Silveira, I., Coutinho, P., 2003. Phenotypes of spinocerebellar ataxia type 6 and familial hemiplegic migraine caused by a unique CACNA1A missense mutation in patients from a large family. Arch. Neurol. 60, 610–614.

Berridge, M.J., Lipp, P., Bootman, M.D., 2000. The versatility and universality of calcium signalling. Nat. Rev. Mol. Cell Biol. 1, 11–21.

Bliss, T.V., Collingridge, G.L., 1993. A synaptic model of memory: long-term potentiation in the hippocampus. Nature 361, 31–39.

Bourtchuladze, R., Frenguelli, B., Blendy, J., Cioffi, D., Schutz, G., Silva, A.J., 1994. Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein. Cell 79, 59–68.

Cemal, C.K., Carroll, C.J., Lawrence, L., Lowrie, M.B., Ruddle, P., Al-Mahdawi, S., King, R.H., Pook, M.A., Huxley, C., Chamberlain, S., 2002. YAC transgenic mice carrying pathological alleles of the MJD1 locus exhibit a mild and slowly progressive cerebellar deficit. Hum. Mol. Genet. 11, 1075–1094.

Day, N.C., Shaw, P.J., McCormack, A.L., Craig, P.J., Smith, W., Beattie, R., Williams, T.L., Ellis, S.B., Ince, P.G., Harpold, M.M., Lodge, D., Volsen, S.G., 1996. Distribution of alpha 1A, alpha 1B and alpha 1E voltage-dependent calcium channel subunits in the human hippocampus and parahippocampal gyrus. Neuroscience 71, 1013–1024.

Denier, C., Ducros, A., Vahedi, K., Joutel, A., Thierry, P., Ritz, A., Castel-novo, G., Deonna, T., Gerard, P., Devoize, J.L., Gayou, A., Perrouty, B., Soisson, T., Autret, A., Warter, J.M., Vighetto, A., Van Bogaert, P., Alam-owitch, S., Roullet, E., Tournier-Lasserve, E., 1999. High prevalence of CACNA1A truncations and broader clinical spectrum in episodic ataxia type 2. Neurology 52, 1816–1821.

Dove, L.S., Nahm, S.S., Murchison, D., Abbott, L.C., Griffith, W.H., 2000. Altered calcium homeostasis in cerebellar Purkinje cells of leaner mutant mice. J. Neurophysiol. 84, 513–524.

Doyle, J., Ren, X., Lennon, G., Stubbs, L., 1997. Mutations in the Cacnl1a4 calcium channel gene are associated with seizures, cerebellar degenera-tion, and ataxia in tottering and leaner mutant mice. Mamm. Genome 8, 113–120.

Dunwiddie, T.V., Lynch, G., 1979. The relationship between extracellu-lar calcium concentrations and the induction of hippocampal long-term potentiation. Brain Res. 169, 103–110.

Fitzjohn, S.M., Collingridge, G.L., 2002. Calcium stores and synaptic plas-ticity. Cell Calcium 32, 405–411.

Fletcher, C.F., Lutz, C.M., O'Sullivan, T.N., Shaughnessy Jr., J.D., Hawkes, R., Frankel, W.N., Copeland, N.G., Jenkins, N.A., 1996. Absence epilepsy in tottering mutant mice is associated with calcium channel defects. Cell 87, 607–617.

Frank, T.C., Nunley, M.C., Sons, H.D., Ramon, R., Abbott, L.C., 2003. Fluoro-jade identification of cerebellar granule cell and purkinje cell death in the alpha1A calcium ion channel mutant mouse, leaner. Neuro-science 118, 667–680.

Grover, L.M., Teyler, T.J., 1990. Two components of long-term potentiation induced by different patterns of afferent activation. Nature 347, 477–479.

Guy, J., Hendrich, B., Holmes, M., Martin, J.E., Bird, A., 2001. A mouse Mecp2-null mutation causes neurological symptoms that mimic Rett syndrome. Nat. Genet. 27, 322–326.

Herrup, K., Wilczynski, S.L., 1982. Cerebellar cell degeneration in the leaner mutant mouse. Neuroscience 7, 2185–2196.

Kaja, S., van de Ven, R.C., Broos, L.A., Frants, R.R., Ferrari, M.D., van den Maagdenberg, A.M., Plomp, J.J., 2007. Characterization of acetylcholine release and the compensatory contribution of non-Ca(v)2.1 channels at motor nerve terminals of leaner Ca(v)2.1-mutant mice. Neuroscience 144, 1278–1287.

Kang, H., Sun, L.D., Atkins, C.M., Soderling, T.R., Wilson, M.A., Tone-gawa, S., 2001. An important role of neural activity-dependent CaMKIV signaling in the consolidation of long-term memory. Cell 106, 771–783.

Katoh, A., Jindal, J., Raymond, J., 2007. Motor deficits in homozygous and heterozygous P/Q-type calcium channel mutants. J. Neurophysiol. 97, 1280–1287.

Kors, E.E., Haan, J., Giffin, N.J., Pazdera, L., Schnittger, C., Lennox, G.G., Terwindt, G.M., Vermeulen, F.L., Van den Maagdenberg, A.M., Frants, R.R., Ferrari, M.D., 2003. Expanding the phenotypic spectrum of the CACNA1A gene T666M mutation: a description of 5 families with familial hemiplegic migraine. Arch. Neurol. 60, 684–688.

Lau, F.C., Abbott, L.C., Rhyu, I.J., Kim, D.S., Chin, H., 1998. Expression of calcium channel alpha1A mRNA and protein in the leaner mouse (tgla/tgla) cerebellum. Brain Res. Mol. Brain Res. 59, 93–99.

Lau, F.C., Frank, T.C., Nahm, S.S., Stoica, G., Abbott, L.C., 2004. Postnatal apoptosis in cerebellar granule cells of homozygous leaner (tg1a/tg1a) mice. Neurotox. Res. 6, 267–280.

Lee, A., Wong, S.T., Gallagher, D., Li, B., Storm, D.R., Scheuer, T., Catterall, W.A., 1999. Ca2+/calmodulin binds to and modulates P/Q-type calcium channels. Nature 399, 155–159.

Lorenzon, N.M., Lutz, C.M., Frankel, W.N., Beam, K.G., 1998. Altered calcium channel currents in Purkinje cells of the neurological mutant mouse leaner. J. Neurosci. 18, 4482–4489.

Mangiarini, L., Sathasivam, K., Seller, M., Cozens, B., Harper, A., Hether-ington, C., Lawton, M., Trottier, Y., Lehrach, H., Davies, S.W., Bates, G.P., 1996. Exon 1 of the HD gene with an expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice. Cell 87, 493–506.

Manto, M.U., 2005. The wide spectrum of spinocerebellar ataxias (SCAs). Cerebellum 4, 2–6.

McManamny, P., Chy, H.S., Finkelstein, D.I., Craythorn, R.G., Crack, P.J., Kola, I., Cheema, S.S., Horne, M.K., Wreford, N.G., O'Bryan, M.K., De Kretser, D.M., Morrison, J.R., 2002. A mouse model of spinal and bulbar muscular atrophy. Hum. Mol. Genet. 11, 2103–2111.

Meier, H., MacPike, A.D., 1971. Three syndromes produced by two mutant genes in the mouse. Clinical, pathological, and ultrastructural bases of tottering, leaner, and heterozygous mice. J. Hered. 62, 297–302.

Mori, Y., Wakamori, M., Oda, S., Fletcher, C.F., Sekiguchi, N., Mori, E., Copeland, N.G., Jenkins, N.A., Matsushita, K., Matsuyama, Z., Imoto,

K., 2000. Reduced voltage sensitivity of activation of P/Q-type Ca2+ channels is associated with the ataxic mouse mutation rolling Nagoya (tg(rol)). J. Neurosci. 20, 5654–5662.

Ophoff, R.A., Terwindt, G.M., Vergouwe, M.N., van Eijk, R., Oefner, P.J., Hoffman, S.M., Lamerdin, J.E., Mohrenweiser, H.W., Bulman, D.E., Ferrari, M., Haan, J., Lindhout, D., van Ommen, G.J., Hofker, M.H., Ferrari, M.D., Frants, R.R., 1996. Familial hemiplegic migraine and episodic ataxia type-2 are caused by mutations in the Ca2+ channel gene CACNL1A4. Cell 87, 543–552.

Schwaller, B., Meyer, M., Schiffmann, S., 2002. 'New' functions for 'old' proteins: the role of the calcium-binding proteins calbindin D-28k, calretinin and parvalbumin, in cerebellar physiology. Studies with knockout mice. Cerebellum 1, 241–258.

Sidman, R., Green, M., Appel, S., 1965. Catalog of the Neurological Mutants of the Mouse. Harvard UP, Cambridge, MA.

Tsuji, S., Meier, H., 1971. Evidence for allelism of leaner and tottering in the mouse. Genet. Res. 17, 83–88.

Volsen, S.G., Day, N.C., McCormack, A.L., Smith, W., Craig, P.J., Beattie, R., Ince, P.G., Shaw, P.J., Ellis, S.B., Gillespie, A., et al., 1995. The expression of neuronal voltage-dependent calcium channels in human cerebellum. Brain Res. Mol. Brain Res. 34, 271–282.

Wakamori, M., Yamazaki, K., Matsunodaira, H., Teramoto, T., Tanaka, I., Niidome, T., Sawada, K., Nishizawa, Y., Sekiguchi, N., Mori, E., Mori, Y., Imoto, K., 1998. Single tottering mutations responsible for the neuropathic phenotype of the P-type calcium channel. J. Biol. Chem. 273, 34857–34867.

Watase, K., Zoghbi, H.Y., 2003. Modelling brain diseases in mice: the challenges of design and analysis. Nat. Rev. Genet. 4, 296–307.

Wise, T.L., Pravtcheva, D.D., 2004. Oligosyndactylism mice have an inversion of chromosome 8. Genetics 168, 2099–2112.

Zhuchenko, O., Bailey, J., Bonnen, P., Ashizawa, T., Stockton, D.W., Amos, C., Dobyns, W.B., Subramony, S.H., Zoghbi, H.Y., Lee, C.C., 1997. Autosomal dominant cerebellar ataxia (SCA6) associated with small polyglutamine expansions in the alpha 1A-voltage-dependent calcium channel. Nat. Genet. 15, 62–69.

Zwingman, T.A., Neumann, P.E., Noebels, J.L., Herrup, K., 2001. Rocker is a new variant of the voltage-dependent calcium channel gene Cacna1a. J. Neurosci. 21, 1169–1178.