Knock-in mice for the R50X mutation in the PYGM gene present with McArdle disease

Gisela Nogales-Gadea,1,2,3,* Tomàs Pino´s,1,3,* Alejandro Lucia,4 Joaquín Arenas,3,5 Yolanda Camara,1,3 Astrid Brull,1,3 Noemí de Luna,1,3 Miguel A. Martín,3,5 Elena Garcia-Arumí,1,3 Ramon Martí1,3,* and Antoni L. Andreu 1,3,*

1 Laboratori de Patologia Mitocandrial i Neuromuscular, Hospital Universitari Vall d’Hebron, Institut de Recerca (VHIR), Universitat Autònoma de Barcelona, 08035 Barcelona, Spain
2 Laboratory of Neuromuscular diseases, Institut de Recerca Hospital de la Santa Creu i Sant Pau-IIB Sant Pau, Universitat Autònoma de Barcelona, 08025 Barcelona, Spain
3 Centre for Biomedical Network Research on Rare Diseases (CIBERER), Instituto de Salud Carlos III, Spain
4 Department of Physiology, Universidad Europea de Madrid, 28670 Villaviciosa de Odón, Madrid, Spain
5 Centro de Investigación, Hospital Universitario 12 de Octubre, 28041 Madrid, Spain

*These authors contributed equally to this work.

Correspondence to: Antoni L. Andreu, MD, Ph.D,
Institut de Recerca Hospital Universitari Vall d’Hebron, Barcelona, Spain,
E-mail: toniandreu.bcn@gmail.com

McArdle disease (glycogenosis type V), the most common muscle glycogenosis, is a recessive disorder caused by mutations in PYGM, the gene encoding myophosphorylase. Patients with McArdle disease typically experience exercise intolerance manifested as acute crises of early fatigue and contractures, sometimes with rhabdomyolysis and myoglobinuria, triggered by static muscle contractions or dynamic exercises. Currently, there are no therapies to restore myophosphorylase activity in patients. Although two spontaneous animal models for McArdle disease have been identified (cattle and sheep), they have rendered a limited amount of information on the pathophysiology of the disorder; therefore, there have been few opportunities for experimental research in the field. We have developed a knock-in mouse model by replacing the wild-type allele of Pygm with a modified allele carrying the common human mutation, p.R50X, which is the most frequent cause of McArdle disease. Histochemical, biochemical and molecular analyses of the phenotype, as well as exercise tests, were carried out in homozygotes, carriers and wild-type mice. p.R50X/p.R50X mice showed undetectable myophosphorylase protein and activity in skeletal muscle. Histochemical and biochemical analyses revealed massive muscle glycogen accumulation in homozygotes, contrast to heterozygotes or wild-type mice, which did not show glycogen accumulation in this tissue. Additional characterization confirmed a McArdle disease-like phenotype in p.R50X/p.R50X mice, i.e. they had hyperCKaemia and very poor exercise performance, as assessed in the wire grip and treadmill tests (6% and 5% of the wild-type values, respectively). This model represents a powerful tool for in-depth studies of the pathophysiology of McArdle disease and other neuromuscular disorders, and for exploring new therapeutic approaches for genetic disorders caused by premature stop codon mutations.

Keywords: McArdle disease; knock-in mouse; p.R50X mutation; glycogenosis type V; neuromuscular disorders
Introduction

Glycogenosis type V [glycogen storage disease type V (GSD V), McArdle disease or myophosphorylase deficiency; OMIM® database number 232600] is an autosomal recessive disorder of muscle glycogen metabolism described by Brian McArdle in 1951 (McArdle, 1951). Patients harbour pathogenic mutations in both alleles of the \textit{PYGM} gene, encoding myophosphorylase, the skeletal muscle isoform of glycogen phosphorylase (Lucia et al., 2008). Myophosphorylase initiates the breakdown of muscle glycogen by removing 1,4-\(\alpha\)-glucosyl units from its outer branches, leading to liberation of glucose-1-phosphate; thus, patients are unable to obtain energy from their muscle glycogen stores.

In virtually all patients, the clinical presentation is dominated by exercise intolerance in the form of reversible, acute crises of early fatigue and contractures, which can be accompanied (in \textasciitilde50\% of cases) by rhabdomyolysis, as reflected by marked increases in serum levels of creatine kinase or myoglobinuria (Lucia et al., 2012). Owing to blocked glycogenolysis, exercise intolerance is more marked during exercise tasks involving anaerobic or aerobic glycolysis for ATP production in muscles. Thus, ‘muscle crises’ can be triggered by: (i) ‘static’ (or isometric) contractions relying on small muscle groups and anaerobic glycogenolysis, eg. carrying weights; or (ii) dynamic exercise involving large muscle mass and aerobic pathways, e.g. climbing stairs, brisk walking (DiMauro, 2007; Lucia et al., 2012). A significant characteristic of the disease is a high serum level of creatine kinase under basal conditions, that is, in the absence of heavy exercise in the previous few hours or days (Lucia et al., 2008, 2012). Fixed weakness, affecting mostly proximal muscles, can also be present in patients aged \textasciitilde40 years (Nadaj-Pakleza et al., 2009; Quinlivan et al., 2010).

Several reports have described the \textit{PYGM} genotype characteristics of relatively large cohorts of patients (Martin et al., 2001; Bruno et al., 2006; Aquaron et al., 2007; Deschauer et al., 2007; Rubio et al., 2007a, b; Quinlivan et al., 2010; Lucia et al., 2012; Vieitez et al., 2011). The most prevalent \textit{PYGM} mutation in the Caucasian population, e.g. with an allele frequency of \~55\% among all (\textit{n} = 239) diagnosed Spanish patients (Lucia et al., 2012), is a nonsense mutation located in exon 1 (p.R50X). This mutation changes an arginine to a stop codon, resulting in nonsense-mediated decay of the messenger RNA (Nogales-Gadea et al., 2008).

Two spontaneous animal models for McArdle disease have been identified, i.e. in Charolais cattle (Angelos et al., 1995) and Merino sheep (Tan et al., 1997) but they have rendered a limited amount of information on the pathophysiology of the disorder, particularly in terms of its exercise phenotype. This prompted us to develop a genetically modified murine model of McArdle disease (knock-in mouse for the common \textit{PYGM} p.R50X mutation). Here, we present the characterization of the phenotype of this model, including biochemical, molecular and histochemical data together with exercise performance tests. In contrast to what is often observed for other rodent models of neuromuscular disease, these mice recapitulate the main features of the human disorder and therefore constitute a valuable tool for further studies and for testing therapeutic approaches.

Materials and methods

All experimental procedures were approved by the animal care and use committee of the Vall d’Hebron Institut de Recerca (procedure 13/04 CEEA; 35/04/08) and were in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 1 2 3) and the Spanish laws (32/2007 and R.D. 1201/2005).

Generation of \textit{Pygm} p.R50X knock-in mouse

To construct the targeting vector, a 7.3 kb fragment of the \textit{Pygm} gene (ranging nt 6380746–6388054; GeneBank accession number NC_000085.5) was amplified by long PCR from mouse C57BL/6J DNA. The primer used were \textit{Pygm}-F and \textit{Pygm}-R (for primer sequences described in this section, see Supplementary Table 1). The resulting DNA fragment was cloned in the pUC19 vector. The \textit{p.R50X} mutation (c.148A \textasciitilde T nucleotide change, GenBank accession number NM_011224.1) was introduced in exon 1 using a site-directed mutagenesis kit (Stratagene) with the primers \textit{p.R50X}-F and \textit{p.R50X}-R. The \textit{Loxp}-flanked neomycin resistance cassette, under the human phosphoglycerate kinase promoter (\textit{Loxp}-PGK-NEO), was inserted in intron 1, 214 nucleotides after exon 1, and the complete sequence of the targeting vector was verified by full sequencing. Transfection of the 129/SvPas embryonic stem cells and blastocyst injections were performed (genOway). G418 resistant mouse embryonic stem cell clones were screened for homologous recombination by long PCR in both 5’ and 3’ extremes, and confirmed by Southern blot analysis. The primers used in the long PCR screening at the 5’ site of the \textit{Pygm} region were L5-F and L5-R; and at the 3’ site were L3-F and L3-R. Eight recombinant clones out of 227 analysed were sequenced to rule out the presence of additional mutations introduced by PCR-amplification. One recombinant clone, containing the \textit{p.R50X} mutation but no other nucleotide changes, was selected for the injection into C57BL/6J blastocysts, which were then re-implanted into pseudo-pregnant females and allowed to develop to term. Two highly chimeric males were selected from the resulting offspring and mated to C57BL/6J females. The resulting pups were studied for germinal line transmission of the recombination event by using the PCR strategy described above. The presence of the \textit{p.R50X} mutation was further verified by sequencing. Heterozygous males were mated with B6.FVB-Tg(Elia cre)C5379Lmgd/J females (Jackson Laboratories), to remove the \textit{loxP}-PGK NEO cassette (this strain targets expression of Cre recombination to the early mouse embryo). The elimination of the cassette in the offspring was analysed by PCR with the primers \textit{LoxP}-F and \textit{LoxP}-R. Homozygous mutant mice were obtained by inter-crossing heterozygous littersmates.

In the experiments described below, animals were killed by cervical dislocation to collect tissue samples. We studied 2-month-old female and male littermates for all the experiments except the plasma creatine kinase determination, which was assessed in 5-month-old mice.

Biochemical and molecular studies

\textit{Glycogen phosphorylase activity}

Gastrocnemius muscle samples were homogenized in 20 volumes (1 ml/50 mg tissue) of cold homogenization buffer (40 mM \(\beta\)-glycerophosphate, 40 mM NaF, 10 mM EDTA and 20 mM \(\beta\)-mercaptoethanol, pH 6.8) and centrifuged at 10000 g for 10 min, at 4°C. Glycogen
phosphorylase activity was measured in the supernatant using a spectrophotometric kinetic method as previously described (Miranda et al., 1981).

**Glycogen determination**

The glycogen content in muscle was measured as previously described (Lo et al., 1970). Briefly, ~150 mg of tissue was boiled for 30 min with 30% KOH, then 1.2 volumes of 95% ethanol were added to precipitate the glycogen. After a centrifugation step (840 g, 25 min), the pelleted glycogen was resuspended in 0.1 ml of water and treated with 0.5 ml of H2SO4 (to hydrolyse glycogen to glucose) plus 0.1 ml of 5% trichloracetic acid (TCA). The mixture was allowed to stand for 30 min at room temperature and then the released glucose was measured spectrophotometrically at 490 nm. A standard curve made with glycogen purified from rabbit liver (Sigma-Aldrich), ranging 0.1–0.8 mg/ml was prepared in parallel. The results were expressed as milligrams of glycogen per gram of tissue.

**Western blot analysis**

Protein extracts of gastrocnemius muscle, obtained as indicated above for glycogen phosphorylase activity, were resolved on 10% SDS-PAGE gels transferred to a polyvinylidene difluoride membrane and probed with a primary antibody against muscle phosphorylase (kindly provided by Prof. Martinuzzi) and an anti-α-tubulin mouse monoclonal antibody (Sigma-Aldrich). Suitable horseradish peroxidase-conjugated secondary antibodies were used and images were obtained with films (FujiFilm) and quantified with NIH ImageJ (version 1.37) software (Scion image, NIH).

**RNA analysis**

Total RNA was obtained from gastrocnemius muscle as previously described (Nogales-Gadea et al., 2008), and from brain and heart tissue following the manufacturer instructions of TRIzol® (Invitrogen). RNA was treated with DNase I, amplification grade (Invitrogen) to eliminate any traces of DNA. The amount and integrity of the RNA was assessed by capillary electrophoresis using the RNA 6000 Nano Chip kit (Agilent Technologies). The levels of RNA degradation were always negligible (data not shown). Complementary DNA was synthesized from RNA using the high-capacity complementary DNA archive kit (Applied Biosystems), which uses random primers. We used real-time PCR, with TaqMan® fluorogenic probes in a 7500 Real-Time PCR System (Applied Biosystems) to assess muscle, heart and liver messenger RNA levels of:

- (i) *Pygm* gene (Mm00478582_m1);
- (ii) glycogen phosphorylase, brain isoform (*Pygb*) gene (Mm00464080_m1); and
- (iii) glycogen phosphorylase, liver isoform (*Pygl*) gene (Mm00500078_m1). Results were normalized to peptidylprolyl isomerase A (cyclophilin A, *Ppia*) gene messenger RNA levels (probe Mm02342430_g1).

**Plasma creatine kinase activity**

Anticoagulated blood was collected from the saphenous vein and diluted 1:3 with PBS, centrifuged at 3000g for 5 min at 4°C and the supernatant (diluted plasma) was immediately used to determine creatine kinase activity using a standard spectrophotometric kinetic method.

**Myoglobinuria assessment**

Urine (~50 µl) was collected from each mouse before and 3 h after performing on the treadmill, and myoglobin was determined by a commercially available ELISA (Mouse Myoglobin ELISA, Life Diagnostics, Inc.) following the manufacturers’ instructions. Samples were 1:3 diluted, mixed with the polyclonal antibody anti-myoglobin conjugated to horseradish peroxidase and added to the plate coated with immobilized monoclonal antibody anti-myoglobin. After 1 h of incubation, wells were washed and the peroxidase reaction was developed by adding the substrate. Absorbance was measured at 450 nm and the results interpolated in a calibration curve ranging from 16 to 250 ng myoglobin/ml.

**Histochemical analysis**

Gastrocnemius, biceps femoris and soleus muscle samples were fixed in 0.1% O.C.T. medium in cold methylbutane for 30 s in liquid nitrogen, kept at −80°C until suitable sections on slides were obtained, and frozen at −20°C until analysis. Glycogen phosphorylase activity staining was performed on gastrocnemius muscle only, by incubating muscle sections for 45 min with a solution containing 1% glucose-1-phosphate, 0.2%, AMP, and 0.02% glycogen in 0.1 M sodium acetate buffer, pH 5.6. Sections were washed with water, Lugol’s iodine was applied for 3 min and samples were mounted with Aquatex. Haematoxylin and eosin standard staining was performed in gastrocnemius, biceps femoris and soleus sections. Glycogen content was assessed in gastrocnemius, biceps femoris and soleus sections by periodic acid Schiff staining, by sequentially incubating the sections with: periodic acid, Schiff solution, haematoxylin and alcohol-xytol dehydration with DPX mounting medium (Sigma-Aldrich). Stained sections were analysed and images obtained with an inverted microscope (Olympus IX 71 Inverted Microscope, Olympus Corporation). Glycogen accumulation in p.R50X/p.R50X mice was morphometrically quantified in type I and II fibres as follows. Consecutive 20-µm thick gastrocnemius sections were stained with periodic acid Schiff (see above) and with a type I-specific myosin heavy chain antibody, respectively. For the latter, sections were incubated for 5 min at room temperature in acetone, washed three times in PBS and incubated for 1 h at room temperature with the mouse monoclonal myosin heavy chain (slow) primary antibody (dilution 1:20; Leica Microsystems). Sections were then washed three times in PBS and incubated with rabbit anti-mouse Alexa-Fluor® 488 secondary antibody (1:200 dilution; Life Technologies). Finally, sections were washed three times in PBS and mounted with Aquatex® (Merk Chemicals). Glycogen accumulation in type I fibres (fluorescent) and type II fibres (non-fluorescent) was quantified in the corresponding fibres in the periodic acid Schiff-stained serial section using NIH ImageJ (version 1.37) software (Scion image, NIH).

**Assessment of exercise capacity**

Researchers in charge of assessing exercise phenotypes were blind to mice *Pygm* genotypes.

**Wire grip test**

Mice were picked up by the tail and placed on a metal wire suspended between two upright bars, 50 cm above a padded table (Petraglia et al., 2010). The time the mouse could hold onto the wire with both proximal limbs was recorded, the maximum time allowed being 180 s. When a mouse did not reach this time, it was tested twice and the maximum hanging time was recorded.

**Treadmill running**

Mice were exercised on an enclosed treadmill (Harvard Apparatus, Panlab) supplied with an electrified grid at the rear of the belt to provide motivation [shocks of 0.2 mA (11 Hz, 200 ms)]. Animals were familiarized with treadmill exercise the day before testing. The day of the test treadmill initial speed was 15 cm/s for 2 min, followed by 2 min at 30 cm/s, and thereafter was increased by 10 cm/s every 2 min until exhaustion, i.e. when the mouse remained more than 5 s
on the electrified grid and was unable to continue running at the following speed load (Ayala et al., 2009). During testing, treadmill inclination was kept at 25% as this inclination ensures attaining the maximum possible load for the mouse cardiorespiratory and oxidative system [i.e. attainment of maximal aerobic capacity, also termed ‘maximal oxygen uptake’ (VO2max)], while preventing injuries (Hoydal et al., 2007). We determined the maximum exercise time completed by each mouse as an index of maximum aerobic capacity (Lightfoot et al., 2001).

Statistical analysis

All statistical analyses were performed using the PASW (v. 19.0 for WINDOWS) with α set at 0.05. We compared molecular, biochemical and exercise phenotypes of the three study groups (wild-type, heterozygous or p.R50X/p.R50X mice) using the non-parametric Kruskal–Wallis test. This test allowed us to determine the statistical effect of the study group to which the mice belonged on the results, i.e. ‘group effect’. Other tests used are indicated in the Results section and/or the figure legends. For statistical purposes, undetectable values were considered as zero.

Results

Generation of the p.R50X knock-in mouse

Figure 1A shows the structure of the engineered vector electroporated into mouse embryonic stem cells to generate the knock-in mutant DNA by homologous recombination (refer to ‘Materials and methods’). After G418 selection in cell culture, 227 embryonic stem clones were screened and eight homologous recombiant clones were detected both by long PCR and confirmed by Southern blot analysis (Fig. 1B and C). The clone 3C1, which harboured the p.R50X mutation and no additional mutations, was used for blastocyst injection. Chimeric animals were obtained and germ-line transmission was later confirmed in F1. The PKG-NEO selection marker was eliminated by mating F1 heterozygous males with B6.FVB-Tg(Ela-cre)CS579Lmgd/J females, generating F2 mice containing only the p.R50X mutation (plus the 34 bp loxP fragment) in the knock-in allele. Finally, male and female F2 heterozygotes were mated to obtain homozygote, heterozygote and wild-type mice, whose genotypes were confirmed by PCR and sequencing (Fig. 1D and E).

Absence of glycogen phosphorylase activity in the gastrocnemius of p.R50X/p.R50X mice

Glycogen phosphorylase activity was totally abolished in the gastrocnemius muscle of all p.R50X/p.R50X mice studied, while their heterozygous counterparts had 50% lower enzyme activity compared with wt/wt mice (P = 0.004 for the group effect, Fig. 2A). When the amount of protein was assessed by western blot analysis, the results closely resembled those obtained for glycogen phosphorylase activity (P = 0.011 for the group effect, Fig. 2B). In skeletal muscle, the Pygm messenger RNA levels of p.R50X/p.R50X and heterozygous mice were ~7% and 60%, respectively, of those obtained in their wt/wt counterparts (P = 0.005 for the group effect), whereas we found no group effect on the messenger RNA levels of the other glycogen phosphorylase isofrom genes (Fig. 3A). In the heart, Pygm messenger RNA levels showed the same pattern as in skeletal muscle, i.e. the transcript levels of p.R50X/p.R50X and heterozygous mice were ~6% and 52%, respectively, of those obtained in their wt/wt counterparts (P = 0.012 for the group effect), whereas there was no group effect for Pygb or Pygl messenger RNA levels (Fig. 3B).

Interestingly, considerable messenger RNA levels of the brain isoform (Pygb) were observed in the murine heart (~30% as compared to the Pygm messenger RNA levels). This observation contrasts with the negligible Pygb messenger RNA levels observed in skeletal muscle. In the brain, no group effect was found for Pygm, Pygb or Pygl messenger RNA levels (Fig. 3C).

Histochemical analyses and glycogen accumulation in skeletal muscle

Figure 4A shows the histochemical images for glycogen phosphorylase, haematoxylin and eosin and periodic acid Schiff staining of gastrocnemius sections. This analysis confirmed the absence of glycogen phosphorylase activity in skeletal muscle in p.R50X/p.R50X mice and partial activity in heterozygote carriers. The haematoxylin and eosin staining in p.R50X/p.R50X mice showed alterations in fibre size and shape in which sub-sarcometal deposits could be observed (see arrows). As for periodic acid Schiff staining of glycogen, findings in p.R50X/p.R50X mice were similar to those reported in patients with McArdle disease, i.e. massive sub-sarcometal accumulation of glycogen, yet with glycogen stores also present in the sarcoplasm. Despite the lower glycogen phosphorylase activity compared with wt/wt mice, neither glycogen accumulation nor morphology alterations in muscle fibres were found in heterozygous mice. Similar results were observed in the other muscle groups (soleus and biceps femoris, data not shown). Glycogen accumulation in p.R50X/p.R50X mice was confirmed by biochemical quantitative analysis in gastrocnemius, which showed values between 48 and 58 mg of glycogen/g of tissue (n = 3), which, on average, indicates a 27-fold increase as compared to the glycogen found in both wt/wt and heterozygous mice. Interestingly, no increase of glycogen content was detected in heterozygotes (between 1.6 and 2.6 mg of glycogen/g of tissue; n = 4) compared with wt/wt mice (between 1.4 and 2.6; n = 3).

Morphometric analysis of serial gastrocnemius sections stained for glycogen content (periodic acid Schiff) and type I fibres (immunostaining with anti-myosin heavy chain antibody) revealed a slight but highly significant difference in glycogen accumulation between type I and type II fibres: the glycogen excess was more pronounced in type II fibres (Fig. 4B).

Exercise tests

The p.R50X/p.R50X mice showed a clear phenotype of exercise intolerance, both in the wire grip and exercise tests. In the wire grip, all wild-type mice tested were able to stay on the wire for...
the maximum time established in our experimental design (180 s), while none of the homozygous p.R50X mice tested were able to stay more than 34 s. Six out of these seven mice were unable to stay more than 7 s, which is <4% of the minimum time reached by the wild-type animals. Three out of 11 heterozygous mice did not reach the maximum time, but all resisted more than 41 s, showing a wide variability in performance times (P = 0.001 for the group effect) (Fig. 5A and Supplementary Videos 1 and 2).

When dynamic performance was tested with the treadmill device, the maximum exercise time in p.R50X/p.R50X mice was only ~41% and ~29% of that attained by their heterozygous and wt/wt counterparts, respectively (P = 0.001 for the group effect) (Fig. 5B and Supplementary Videos 3 and 4).

Plasma creatine kinase activity and myoglobinuria

Levels of plasma creatine kinase in p.R50X/R50X mice (median 1137 IU/l; range 336–1875) were much higher than in wt/wt
animals (median 89 IU/l; range 37–181) \((P = 0.009;\) Mann–Whitney U test, \(n = 5\) for each group). In addition, preliminary results indicated that \(p.R50X/p.R50X\) mice develop exercise-induced myoglobinuria: two \(p.R50X/p.R50X\) animals tested had 375 and 1500 ng myoglobin/ml of urine after treadmill exercise, while it was undetectable in three out of three \(wt/wt\) mice and positive in only one (100 ng/ml) out of the four heterozygous mice we tested.

**Discussion**

To our knowledge, this is the first successful attempt to develop a genetically modified animal model of McArdle disease. The observation that the \(p.R50X\) is the most prevalent mutation, at least among Caucasian patients (Martin et al., 2001; Bruno et al., 2006; Aquaron et al., 2007; Deschauer et al., 2007; Rubio et al., 2007a, b; Quinlivan et al., 2010; Vieitez et al., 2011; Lucia et al., 2012) prompted us to develop a knock-in specific mouse model for this common defect rather than a classic strategy where the gene is partially or totally eliminated. The highly specialized function and the high evolutionary conservation of the \(PYGM\) gene sequences (Hudson et al., 1993) made us hypothesize that the genetically modified mouse would present with a phenotype consistent with McArdle disease. This hypothesis was also based on the observation that the two spontaneous animal models identified to date, i.e. Charolais cattle (Angelos et al., 1995) and Merino sheep (Tan et al., 1997), showed features present in patients such as rhabdomyolysis or exercise intolerance. We thus expected our model to reflect the main characteristics present in patients.

Knock-in mice homozygous for the common \(p.R50X\) human mutation showed absence of glycogen phosphorylase activity in muscle and subsequent blocked glycogenolysis, which resulted in a marked exercise intolerance that closely resembles the clinical presentation of the disease. Muscle biochemical and molecular
analyses also showed the common features of the disease. As in patients harbouring the p.R50X mutation (Nogales-Gadea et al., 2008), the muscle Pygm transcripts of knock-in mice undergo an extensive degree of ‘nonsense-mediated decay’; indeed, messenger RNA levels of p.R50X/p.R50X mice were only ~10% of those found in wt/wt mice. Such low transcript levels are unlikely to have a translational effect on the protein levels; and, if translated, the resulting protein would be truncated and therefore unstable, as confirmed by western blot analyses showing absence of protein levels in all p.R50X/p.R50X mice. Blocked glycogenolysis resulted in massive accumulation of glycogen in the gastrocnemius muscle, not only subsarcolemmal, as typically described in patients (Nadaj-Pakleza et al., 2009) but also intrasarcoplasmic. In fact, the increase of muscle glycogen content observed in p.50X/p.50X mice (27-fold) was actually much higher than that observed in patients with McArdle disease, which rarely increases more than 2-fold (Nielsen et al., 2002). This marked glycogen accumulation might be explained, at least partly, by the fact that mouse fast fibres have a greater shift towards glycolytic metabolism than humans and mouse muscle has a much higher proportion of fast, glycolytic fibres (Agbulut et al., 2003) [e.g. there are only 1–8% of slow oxidative fibres in mouse

Figure 4 (A) Representative images obtained from histochemical analyses of gastrocnemius muscle. GP = glycogen phosphorylase activity staining; HE = haematoxylin–eosin staining and PAS = periodic acid Schiff staining. The black bar in the wt/wt glycogen phosphorylase image represents 200 μm; the other images were obtained at the same magnification. Arrows in the haematoxylin and eosin image of the homozygote mouse indicate glycogen accumulation. (B) Morphometric quantification of the periodic acid Schiff staining of type I and type II fibres in gastrocnemius sections of two different homozygous p.R50X mice. n indicates the total number of fibres counted for each set of values. For each mouse, the results are expressed as a percentage, taking as 100% the mean value obtained for the type I fibres. Error bars indicate standard deviations. For both mice, the mean periodic acid Schiff intensity of type II fibres (normalized by fibre surface) was significantly higher than that of the type I fibres (P < 0.001; Student’s t-test). wt = wild-type.
Knock-in mouse model for McArdle disease

**Figure 5** Results of the exercise tests. (A) Wire grip test: the maximum time that the mouse could hold onto the wire with both proximal limbs is represented. (B) Treadmill test: the maximum exercise time completed by each mouse is represented. Open circles = females, filled circles = males.

gastrocnemius muscle versus a more balanced proportion of fast versus slow twitch in humans (Kho et al., 2006). Histochemical analysis showed a similar pattern of glycogen accumulation (both at the subsarcolemmal and sarcoplasmic level in other muscles of p.R50X/p.R50X mice, i.e. biceps femoris or soleus muscle (the latter having 58% type I fibres in mice) (Kho et al., 2006). Interestingly, the glycogen accumulation was more pronounced in less-oxidative (type II) fibres, which are more dependent on glycogen metabolism.

Patients typically exhibit intolerance to static and dynamic exercises. During static or isometric exercise, e.g. in patients carrying a heavy weight, or handgrip exercise and, in rodent models hanging onto a grip, high mechanical demands are imposed on a relatively small muscle mass, and the sustained muscular contraction increases the pressure inside the muscle, causing the supply of oxygenated blood to be transiently cut off (DiMauro, 2007; Lucia et al., 2008). In this situation, muscles rely on an anaerobic energy supply from intracellular glycogen stores. The exercise intolerance phenotype of p.R50X/p.R50X mice was in fact most marked in the wire grip test (muscle performance <10% of that reached by both heterozygous and wt/wt mice). On the other hand, dynamic, ‘aerobic’ exercises involving larger muscle mass and smaller mechanical loads, e.g. in human patients, stair-climbing or very brisk walking and in model rodents forced treadmill running, can also trigger acute exercise intolerance. Indeed, muscle oxidative capacity in patients is impaired (Zange et al., 2003) because glycolytic flux is reduced and thus their ability to produce pyruvate is severely reduced (Lucia et al., 2008). In p.R50X/p.R50X mice, maximal aerobic capacity (as determined by maximal treadmill exercise time) was only ~29% of the mean value obtained in wt/wt mice. This is consistent with recent data on the largest series of patients available (Lucia et al., 2012) where the mean maximal aerobic capacity (expressed as VO2max) was very low, averaging 18.2 ml O2/kg/min, which is clearly below the minimum threshold for optimal health (28 ml O2/kg/min; Lucia et al., 2008).

Besides molecular genetics and biopsy analysis, basal hyperCKaemia is the main laboratory diagnostic feature indicative of McArdle disease (Lucia et al., 2008), as recently confirmed in a large series of Spanish patients (Lucia et al., 2012). HyperCKaemia is usually more marked in males than females (~2 times higher mean values in the former, likely owing to their higher muscle mass), yet with large interindividual variability (Mate-Munoz et al., 2007). All these phenomena were corroborated in our model: despite individual variability, mean basal serum creatine kinase activity was 13-fold higher in p.R50X/p.R50X than in wt/wt mice. In addition, the occurrence of myoglobinuria after exercise in our model also points to increased muscle fragility in p.R50X/p.R50X mice. These and other molecular, metabolic and physical aspects of the model will have to be more extensively studied when homogeneous genetic background has been established through backcrossing, a process that is currently underway.

An interesting finding of this study is the observation of lower exercise capacity of heterozygous mice compared with wt/wt mice. This result contrasts with the autosomal recessive nature of the disease in humans with individuals carrying only one mutant PYGM allele being asymptomatic (Andersen et al., 2006), and with the recent observation that, compared with ‘typical’ patients with McArdle disease, two patients with minimal residual myophosphorylase had a much milder form of the disease (Vising et al., 2009). In our murine model there was a clear reduction of glycogen phosphorylase activity in the heterozygotes (50% reduced glycogen phosphorylase activity in muscle compared with wt/wt mice), but this phenomenon did not result in glycogen accumulation or fragile fibres (i.e. no exercise-induced myoglobinuria or increased basal creatine kinase). This might suggest that in heterozygous mice partially compromised exercise performance is more related to partial metabolic limitations rather than to myopathy-induced structural alterations. Further research is, however, necessary to elucidate the reasons why exercise capacity appears to be more affected in heterozygous mice compared with heterozygous humans.
In contrast to available rodent models for other neuromuscular diseases, which do not show a human-like phenotype, here we present a murine model for the most common mutation, p.R50X, causing McArdle disease, which presents with the main features of the human phenotype. This model may represent a valid tool for in-depth studies of the pathophysiology of this disorder, as well as for new therapeutic approaches for this and other neuromuscular diseases. Interestingly, because p.R50X is a nonsense mutation affecting only one tissue whose function can be finely evaluated, our model could also be used for exploring novel pharmacological approaches based on read-through strategies targeting premature termination codon mutations.

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Supplementary material

Supplementary material is available at Brain online.

References


