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Brunetti, Dario; Dusi, Sabrina; Giordano, Carla; Lamperti, Costanza; Morbin, Michela; Fugnanesi, Valeria; Marchet, Silvia; Fagioli, Gigliola; Sibon, Ody; Moggio, Maurizio
Published in: Brain
DOI: 10.1093/brain/awt325

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Document Version
Publisher's PDF, also known as Version of record

Publication date: 2014

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Pantethine treatment is effective in recovering the disease phenotype induced by ketogenic diet in a pantothenate kinase-associated neurodegeneration mouse model

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Pantothenate kinase-associated neurodegeneration, caused by mutations in the PANK2 gene, is an autosomal recessive disorder characterized by dystonia, dysarthria, rigidity, pigmentary retinal degeneration and brain iron accumulation. PANK2 encodes the mitochondrial enzyme pantothenate kinase type 2, responsible for the phosphorylation of pantothenate or vitamin B5 in the biosynthesis of co-enzyme A. A Pank2 knockout (Pank2−/−) mouse model did not recapitulate the human disease but showed azoospermia and mitochondrial dysfunctions. We challenged this mouse model with a low glucose and high lipid content diet (ketogenic diet) to stimulate lipid use by mitochondrial beta-oxidation. In the presence of a shortage of co-enzyme A, this diet could evoke a general impairment of bioenergetic metabolism. Only Pank2−/− mice fed with a ketogenic diet developed a pantothenate kinase-associated neurodegeneration-like syndrome characterized by severe motor dysfunction, neurodegeneration and severely altered mitochondria in the central and peripheral nervous systems. These mice also showed structural alteration of muscle morphology, which was comparable with that observed in a patient with pantothenate kinase-associated neurodegeneration. We here demonstrate that pantethine administration can prevent the onset of the neuromuscular phenotype in mice suggesting the possibility of experimental treatment in patients with pantothenate kinase-associated neurodegeneration.

Keywords: pantothenate kinase-associated neurodegeneration (PKAN); mitochondria; ketogenic diet; pantethine
Abbreviation: PKAN = pantothenate kinase-associated neurodegeneration
Introduction

The common feature of a group of genetic disorders, termed neurodegeneration with brain iron accumulation, is brain iron overload identified by radiological and histopathological examinations (Krueer et al., 2012). Different subtypes of neurodegeneration with brain iron accumulation have been defined at the genetic level but pantothenate kinase-associated neurodegeneration (PKAN) syndrome is the most frequent form.

PKAN is caused by mutations in the PANK2 gene, which codes for the mitochondrial enzyme pantothenate kinase 2. This enzyme is involved in the co-enzyme A biosynthetic pathway, catalysing the phosphorylation of vitamin B5 or pantothenate (Hayflick, 2003). PKAN usually manifests in childhood with gait disturbances and rapidly progresses to a severe movement deficit with dystonia, dysarthria and dysphagia. The hallmark of this disease is the eye-of-the-tiger signal in the globus pallidus on T2-weighted MRI (Hayflick et al., 2003; Gregory et al., 2009).

To date, the mechanistic connection linking PANK2 dysfunction, neurodegeneration and alteration of iron homeostasis has not been understood, thus preventing our comprehension of the pathogenesis of the disease and the design of efficient therapeutic strategies. It has been proposed that reduced PANK2 enzymatic activity determines the accumulation of cysteine, which may chelate iron thus promoting the formation of free radicals (Gregory et al., 2008); alternatively, defects in co-enzyme A and, as a consequence, in phospholipid metabolism may damage the membranes and lead to increased oxidative stress, which may alter iron homeostasis (Leonardi et al., 2007).

The mouse models of PKAN display incomplete phenotypes, including hardly any brain iron accumulation. Pank2−/− mice show growth reduction, retinal degeneration, male infertility because of azoospermia (Kuo et al., 2005), and mitochondrial dysfunctions (Brunetti et al., 2012) under standard diet conditions. Retinal degeneration (Kuo et al., 2005) was not confirmed in a recent Pank2 knockout mouse (Garcia et al., 2012) and this phenotype is uncertain. A movement disorder was present in mice on a pantothenic acid-deficient diet (Kuo et al., 2007). A Pank1 knockout mouse (Leonardi et al., 2010) displayed a metabolic disorder characterized by altered fatty acid oxidation and gluconeogenesis, causing mild hypoglycaemia. An additional mouse model consisting of a double Pank1/Pank2 knockout (Garcia et al., 2012) showed a severe phenotype characterized by hypoglycaemia and hyperketonaemia leading to dysfunctional post-natal development and premature death at 17 days.

Based on the role of co-enzyme A in several crucial cellular metabolic pathways, we tested the hypothesis to stress the Pank2−/− mouse model with a high-fat ketogenic diet. Ketone bodies produced by the ketogenic diet through fatty acid oxidation bypass glycolysis and enter the citric acid cycle to produce oxidative phosphorylation (OXPHOS) substrate. Mice on a ketogenic diet use mainly fatty acid oxidation and OXPHOS for ATP production as compared to mice on a standard diet (Laffel, 1999). We observed that only ketogenic diet-fed Pank2−/− mice presented typical signs of neurological and motor impairment, as well as neuropathological findings, resembling the phenotype observed in patients with PKAN.

Moreover, these mice showed muscular dysfunctions with mitochondrial morphological alterations, which were also detected in the muscle of a patient with PKAN.

Recently, a PANK2 knockout Drosophila model has shown that pantethine can serve as a compound to bypass the block due to severe impairment of pantothenate kinase and that it is able to rescue brain degeneration, mitochondrial dysfunction and locomotor disabilities (Rana et al., 2010). To determine if pantethine was able to counteract the disease phenotype elicited in ketogenic diet-fed Pank2−/− mice, we continuously administered pantethine in drinking water during the ketogenic treatment. Our data indicated that pantethine treatment was safe, with no side effects and was able to ameliorate both the majority of the symptoms in the nervous and muscular systems and the morphological features of neuronal and mitochondrial damage.

Materials and methods

Animals and diets

Animal studies were approved by the Ethics Committee of the Foundation IRCCS Neurological Institute C. Besta, in accordance with guidelines of the Italian Ministry of Health: Project no. BT4/2011. The use and care of animals followed the Italian Law D.L. 116/1992 and the EU directive 86/609/CEE.

Standard diet (Mucedola), ketogenic diet (E15149-30, ssniff Spezialdiaten) and water were given ad libitum. Ketogenic diet composition: 79.2 % fat; 8% protein; 5% crude fibre; 45.5 % crude ash; 0.6% starch; 0.7% sugar (31.6 MJ/kg), with multi-vitamin addition. Pantethine (Sigma) was administered at a concentration of 15 mg/kg/day in drinking water. The JM129/SvJ-C57BL/6 Pank2−/− mice used in this study were kindly provided by Professor Hayflick (Kuo et al., 2005).

Animals were housed two or three in a cage, in a temperature-controlled (21±C) room with a 12 h light-dark cycle and ~60% relative humidity.

The experimental design included four groups of mice: (i) the ‘standard diet’ group composed of eight Pank2−/− mice (four male and four female) and nine Pank2+/− (five male and four female) on a standard diet for 2 months; (ii) the ‘standard diet + pantethine’ group composed of eight Pank2−/− (four male and four female) and 12 Pank2+/− (five male and seven female) on a standard diet with the concomitant administration of pantethine for 2 months; (iii) the ‘ketogenic diet’ group composed of 20 Pank2−/− mice (nine male and 11 female) and 26 Pank2+/− (11 male and 15 female) on an ad libitum ketogenic diet for 2 months; and (iv) the ‘ketogenic diet + pantethine’ group composed of 13 Pank2−/− mice (five male and eight female) and 16 Pank2+/− (five male and 11 female) on an ad libitum ketogenic diet with the concomitant administration of pantethine.

Behavioural and motor skills analysis

The different groups were monitored weekly for onset of postural abnormalities, loss of weight and general behavioural changes. We detected spontaneous motor activity over a continuous period of 15 h (at any time between 17:00 pm and 08:00 am) in an activity cage (Ugo Basile) for four single-gender groups of 3-month-old Pank2−/− and Pank2+/− mice, each comprising three mice of each genotype. In total 12 Pank2+/+ and 12 Pank2−/− mice were analysed.
Measure of motor exercise endurance was evaluated using a treadmill apparatus (Columbus Instruments) counting the number of falls in the motivational grid during a gradually accelerating program with speed initially at 3.8 m/min and increasing by 3 m/min every 2 min. The test was terminated by exhaustion, defined by >10 falls/min into the motivational grid.

A footprint test was performed by painting hindlimbs with non-toxic ink and placing mice at one end of an enclosed, dark tunnel on white paper. Mice walked along a 28 cm long, 7 cm wide strip; stride length and width of consecutive steps were measured. These tests were carried out in 3-month-old mice to monitor the phenotype.

Histology, histochemistry and immunohistochemistry

Histological, histochemical and immunohistochemical analyses were performed on formalin-fixed and paraffin-embedded brain tissues from the following treatment groups: standard diet Pank2+/+ (n = 3); standard diet Pank2+/− (n = 2); ketogenic diet Pank2+/+ (n = 3); ketogenic diet Pank2+/− (n = 3); ketogenic diet + pantethine Pank2+/+ (n = 3); and ketogenic diet + pantethine Pank2+/− (n = 3). Sciatic nerves were surgically removed and processed for epoxy resin embedding as previously described (Brunetti et al., 2012). In particular, each sciatic nerve was cut in proximal, middle and distal segments and dehydrated separately. Ultrastructural analyses were conducted on each stump. Brain ultrastructural analysis was performed on the following treatment groups: standard diet Pank2+/+ (n = 1); standard diet Pank2+/− (n = 1); ketogenic diet Pank2+/+ (n = 3); ketogenic diet Pank2+/− (n = 3); ketogenic diet + pantethine Pank2+/+ (n = 3); and ketogenic diet + pantethine Pank2+/− (n = 3). Sagittally spliced brains were fixed by immersion in glutaraldehyde (2.5% in phosphate buffer). After fixation, serial sagittal slides of 1-mm thick were obtained. Selected areas of interest were sampled, post-fixed in osmium tetroxide and embedded in Epon epoxy resin. Thin sections (80–90 nm) were stained with uranyl acetate and lead citrate and examined with a CM10 Philips electron microscope.

Patient and mouse muscle tissues were fixed in 2.5% glutaraldehyde, processed as previously described (Napoli et al., 2011) and post-fixed in 2% osmium tetroxide for 1 h. After dehydration, the specimens were embedded in epoxy resin. Ultrathin sections were cut and stained with uranyl acetate and lead citrate, then examined with a Zeiss electron microscope.

Results

Mice phenotyping

We constantly monitored the weight of Pank2+/+ and Pank2−/− mice during the administration of standard or ketogenic diet. As also previously reported (Kuo et al., 2005) Pank2−/− mice under standard diet showed a slight weight reduction as compared with Pank2+/+ mice, whereas during ketogenic treatment Pank2−/− mice showed a fast and progressive weight loss (Fig. 1A and B). Moreover, only Pank2−/− mice fed a ketogenic diet manifested kyphosis with hunched position, (Fig. 1C), feet clasping (Fig. 1D), whitening of the fur and rigidity of the tail (Fig. 1E), as well as dystonic limb positioning (Fig. 1F).

We asked whether these signs could have been prevented by pantethine administration. To this aim we treated a group of Pank2+/+ and Pank2−/− mice (‘ketogenic diet + pantethine’ group) with a ketogenic diet and the concomitant administration of pantethine (15 mg/kg/day) in drinking water. This concentration was established based on a dosage of 900 mg/day in an adult individual (n = 14) considering an average weight of 60 kg. We also tested various concentrations of pantethine in mice over 3 weeks and determined that a dose of up to 480 mg/kg/day was tolerated without any side effects on weight and drinking intake (Supplementary Fig. 1).

We observed that, with pantethine administration to mice fed a ketogenic diet, the weight of Pank2−/− mice was similar to that of Pank2+/+ mice (Fig. 1G), no signs of kyphosis or feet clasping were present (Fig. 1H) and body size approached that of Pank2−/− mice.

Electron microscopy analysis

Sciatic nerve analysis was performed on the following treatment groups: standard diet Pank2+/+ (n = 1); standard diet Pank2+/− (n = 1); ketogenic diet Pank2+/+ (n = 3); ketogenic diet Pank2+/− (n = 3); ketogenic diet + pantethine Pank2+/+ (n = 3); and ketogenic diet + pantethine Pank2+/− (n = 3). Sciatic nerves were surgically removed and processed for epoxy resin embedding as previously described (Brunetti et al., 2012). In particular, each sciatic nerve was cut in proximal, middle and distal segments and dehydrated separately.

Ultrastructural analyses were conducted on each stump. Brain ultrastructural analysis was performed on the following treatment groups: standard diet Pank2+/+ (n = 1); standard diet Pank2+/− (n = 1); ketogenic diet Pank2+/+ (n = 3); ketogenic diet Pank2+/− (n = 3); ketogenic diet + pantethine Pank2+/+ (n = 3); and ketogenic diet + pantethine Pank2+/− (n = 3). Sagittally spliced brains were fixed by immersion in glutaraldehyde (2.5% in phosphate buffer). After fixation, serial sagittal slides of 1 mm thick were obtained. Selected areas of interest were sampled, post-fixed in osmium tetroxide and embedded in Epon epoxy resin. Thin sections (80–90 nm) were stained with uranyl acetate and lead citrate and examined with a Zeiss electron microscope.

Evaluation of mitochondrial bioenergetics

We analysed mitochondrial energy metabolism in isolated mitochondria derived from brains of standard diet Pank2+/+ (n = 3); standard diet Pank2+/− (n = 3); ketogenic diet Pank2+/+ (n = 3); ketogenic diet Pank2+/− (n = 3); and standard diet + pantethine Pank2+/+ (n = 3) mice, using an XF96 Extracellular Flux Analyzer (Seahorse Bioscience) as previously reported (Brunetti et al., 2012).

Derivation of neurons from sciatic nerve and analysis of mitochondrial membrane potential

Detection of mitochondrial potential was performed using JC1 staining kit on neurons derived from sciatic nerves isolated from adult mouse as previously described (Brunetti et al., 2012).
fed a standard diet (Fig. 1I). Most importantly, Pank2−/− mice fed a ketogenic diet died after 2 months whereas the administration of pantethine prolonged their survival for up to 5 months (Fig. 1J). We could not verify the recovery of retina degeneration as this initial observation (Kuo et al., 2005) was not confirmed by our own investigations and also not reported in another Pank2 knockout mouse model (Garcia et al., 2012). On the contrary, azoospermia was confirmed in mice fed a standard diet and ketogenic diet but was not rescued by pantethine treatment during the period of our observation and with the dose used.

Motor performance evaluation

Pank2−/− mice fed a ketogenic diet were lethargic and showed a significant reduction of spontaneous movements as compared with Pank2+/+ mice fed a ketogenic diet, whereas no differences were observed when fed a standard diet (Fig. 2A).

Quantitative motor tests revealed significantly lower activity in Pank2−/− mice on a ketogenic diet as compared with Pank2+/+ mice; no differences were evident on a standard diet (Fig. 2B). The footprint patterns were assessed quantitatively by measuring stride length and hind base width. We found that Pank2−/− mice on a ketogenic diet exhibited shorter stride lengths and hind paw width, and an irregular gait as compared with Pank2+/+ mice on a ketogenic diet (Fig. 2C). All of these abnormalities were recovered by pantethine treatment and Pank2−/− mice behaved in the same way as their control littermates (Fig. 2A–C).

Neuropathology of Pank2−/− mice fed a ketogenic diet

On histological and immunohistochemical analysis of the whole brains we did not observe massive neural loss, gliosis or demyelination in Pank2−/− mice on a standard diet or a ketogenic diet (data not shown). However, we noticed the presence of small, scattered groups of neurons with eosinophilic, periodic acid Schiff-positive round cytoplasmic inclusions (Fig. 3A–C). These features were observed only in Pank2−/− mice on a ketogenic diet, and were located mostly in the midbrain, putamen and amygdala. On immunohistochemistry, the inclusions were sharply positive for ubiquitin (Fig. 3D and F) and negative for amyloid precursor protein, phosphorylated tau, α-synuclein, and high molecular weight neurofilaments (not shown). In addition, ubiquitin

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**Figure 1** Mice phenotype. (A) Body weight of Pank2+/+ and Pank2−/− mice fed a standard diet (SD). (B) Body weight of Pank2+/+ and Pank2−/− mice on a ketogenic diet (KD). (C) Kyphosis with hunched position; (D) feet clasp in ketogenic diet-fed Pank2−/− (arrow); (E) whitening of the fur and rigidity of the tail in ketogenic diet-fed Pank2−/−; (F) dystonic hindlimb position. (G) Body weight of Pank2−/− mice was similar to that of Pank2+/+ fed a ketogenic diet with pantethine administration (KD + P). (H) No sign of kyphosis or feet clasp were present in Pank2−/− mice on a ketogenic diet with pantethine administration (arrow). (I) Comparison of Pank2−/− body size in mice on standard (SD), ketogenic (KD) and ketogenic + pantethine (KD + P) diets (J) Survival curves for different diet conditions: Pank2−/− mice fed a ketogenic diet died after 2 months (dotted green line); Pank2+/+ mice fed a ketogenic (dotted yellow line); Pank2+/+ mice fed a ketogenic diet with pantethine (dotted pink line); Pank2−/− mice fed a ketogenic diet with pantethine administration prolonged their survival by up to 5 months (orange line); Pank2−/− mice fed a standard diet (dotted brown line); and Pank2+/+ mice fed a standard diet (blue line). In A, B and G, the red symbols indicate Pank2−/−; blue symbols indicate Pank2+/+.  

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stain highlighted finely granular cytoplasmic deposits in several neurons, as well as large, ubiquitin positive degenerating neurons (Fig. 3D and E). Iron deposits were not observed with Perl’s stain (data not shown).

Pantethine treatment of Pank2+/+ mice on a ketogenic diet led to the disappearance of neuronal cytoplasmic inclusion on haema-toxylin-eosin and periodic acid Schiff stain. Ubiquitin was merely detectable by immunohistochemistry after treatment (Fig. 3G) and

Figure 2 Motor performance evaluation. (A) Activity cage: no differences were observed in Pank2+/+ and Pank2−/− mice on a standard diet (SD) or with pantethine (SD + P); Pank2+/+ mice were more active with pantethine treatment (*P < 0.05, two-tailed, unpaired Student’s t-test). Pank2−/− mice fed a ketogenic diet were lethargic and showed a significant reduction of spontaneous movements as compared with Pank2+/+ mice on a ketogenic diet (**P < 0.001, two-tailed, unpaired Student’s t-test). Pantethine treatment (KD + P) rescues the reduced movements. (B) Treadmill test: no differences in the distance travelled by Pank2+/+ and Pank2−/− mice on a standard diet (SD) or with pantethine (SD + P). Pank2−/− mice on a ketogenic diet ran only 50 m as compared to 180 m of Pank2+/+ mice (**P < 0.001, two-tailed, unpaired Student’s t-test). Pantethine treatment (KD + P) restored the running capability in Pank2−/− mice and increased the performance in Pank2+/+ mice (*P < 0.05, two-tailed, unpaired Student’s t-test). (C) Footprint pattern: Pank2−/− mice fed a ketogenic diet showed shorter stride lengths and hind paw width, and an irregular gait as compared with Pank2+/+ mice fed a ketogenic diet. Pantethine treatment (KD + P) abolishes the movement disorders. In A and B, the red bars indicate Pank2−/− mice and blue bars indicate Pank2+/+ mice, respectively.
Ultrastructural features of central and peripheral nervous systems

Ultrastructural analysis was performed on basal ganglia and peripheral nerve of $\text{Pank}2^{-/-}$ and $\text{Pank}2^{+/+}$ mice under different diet conditions. In the basal ganglia, $\text{Pank}2^{-/-}$ animals on a standard diet showed the presence of numerous mitochondria with abnormal, swollen cristae (Fig. 4). These features were worsened by a ketogenic diet, which led to focal loss of cristae (Fig. 4). $\text{Pank}2^{-/-}$ animals fed a ketogenic diet also showed cytoplasmic deposits of lipofuscin (data not shown). Notably, pantethine administration completely rescued the mitochondrial morphology of $\text{Pank}2^{-/-}$ animals on a standard diet, which were indistinguishable from the wild-type littermates (Fig. 4), and ameliorated the morphology of ketogenic diet-fed $\text{Pank}2^{-/-}$ mice (Fig. 4).

Ultrastructural analysis of peripheral nerve of $\text{Pank}2^{-/-}$ animals on a ketogenic diet showed the presence of swollen mitochondria, characterized by cristae degeneration and by the presence of amorphous material in the matrix (Fig. 5). Pantethine
administration was able to completely rescue the mitochondrial morphology both in the PNS and CNS. No ultrastructural alterations were observed in \( \text{Pank2}^{+/+} \) mice fed with standard or ketogenic diets (Figs 4 and 5).

**Pantethine restores mitochondrial membrane potential of \( \text{Pank2}^{-/-} \) neurons**

To evaluate mitochondrial membrane potential we used JC1 staining. As shown in Fig. 6, neurons derived from \( \text{Pank2}^{+/+} \) animals treated with a standard or ketogenic diet presented red fluorescent aggregates indicating the preservation of the mitochondrial membrane potential. On the contrary, neurons derived from \( \text{Pank2}^{-/-} \) mice treated with a standard or ketogenic diet presented with a diffuse green fluorescence (Fig. 6) confirming the presence of a defective mitochondrial membrane potential. Interestingly, neurons derived from \( \text{Pank2}^{-/-} \) mice under

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**Figure 4** Electron microscopy of basal ganglia in \( \text{Pank2}^{+/+} \) and \( \text{Pank2}^{-/-} \) mice under different diet conditions. The analysed animals were 3 months old. \( \text{Pank2}^{+/+} \) mice on a standard diet (SD), a standard diet + pantethine (SD + P), ketogenic diet (KD) and ketogenic diet + pantethine (KD + P) show normal mitochondria. \( \text{Pank2}^{-/-} \) mice on a standard diet show mitochondria with swollen cristae (asterisks and insert). With a ketogenic diet the mitochondrial morphology is worsened with focal loss of cristae (arrow and insert). \( \text{Pank2}^{-/-} \) mice on standard diet with pantethine were similar to wild-type littermates; with a ketogenic diet with pantethine there is a prevalence of mitochondria with normal cristae morphology, although a few mitochondria still showed swollen cristae.

**Figure 5** Electron microscopy of peripheral nerve in \( \text{Pank2}^{+/+} \) and \( \text{Pank2}^{-/-} \) mice under different diet conditions. The analysed animals were 3 months old. Sciatic nerve axons of \( \text{Pank2}^{+/+} \) mice on a standard diet (SD), standard diet + pantethine (SD + P), ketogenic diet (KD) and ketogenic diet + pantethine (KD + P) show normal mitochondria. Sciatic nerve axons of \( \text{Pank2}^{-/-} \) mice on a standard diet and ketogenic diet show swollen mitochondria with altered cristae (arrows). Sciatic nerve axons of \( \text{Pank2}^{-/-} \) mice on a standard diet + pantethine and ketogenic diet + pantethine show a high prevalence of normally-shaped mitochondria with regular cristae organization (arrowheads). Scale bars = 500 \( \mu \text{m} \).
standard and ketogenic treatment plus the addition of pantethine in drinking water, showed predominantly red fluorescence aggregates indicating that the mitochondrial membrane potential was preserved.

**Pantethine improves mitochondrial respiration**

We evaluated respiration with microscale oxygraphy on mitochondria isolated from brains of Pank2+/+ and Pank2−/− mice treated with pantethine and we compared the results with untreated mice. Measurement was not performed in mitochondria derived from mice under ketogenic treatment because of technical difficulties, probably due to the presence of increased fat levels in the brain. We measured basal oxygen consumption rate, and oxygen consumption rate after ADP addition, and after oligomycin addition. We observed that pantethine was able to significantly increase oxygen consumption rate under all conditions tested in both Pank2+/+ and Pank2−/− mitochondria (Fig. 7). In particular, pantethine determines a doubling in oxygen consumption rate after ADP stimulation suggesting a tightly coupled respiration with ATP production.

These differences were statistically significant as demonstrated by an unpaired, two-sided Student’s t-test, assuming unequal variance. Values for statistical significance were set at \( P < 0.05 \).

**Comparison of muscle derived from Pank2−/− mice on a ketogenic diet versus a patient with pantothenate kinase-associated neurodegeneration**

COX histochemical reaction of the muscle derived from Pank2−/− mice fed a ketogenic diet revealed a peculiar staining pattern, likely because of the presence of abnormal mitochondria (Fig. 8A). We had the opportunity to study the muscle biopsy of a 6-year-old patient with PKAN (PANK2 mutations: N500I + IVS2-1G > A). We did not observe any defects in the respiratory chain enzymatic activities apart from a succinate dehydrogenase (SDH) activity below the lower control value (not shown), but we found the same COX staining pattern (Fig. 8B) observed in mice. To characterize these mitochondria further we performed electron microscopy, which highlighted the presence of giant mitochondria spanning the sarcomere between two neighbouring Z-lines and showing irregularly shaped cristae (Fig. 8C).

The histological alterations of the Pank2−/− muscle highlighted by both trichrome and COX staining were absent in pantethine-treated mice (Fig. 9). Moreover, no histological abnormalities were evident in standard diet or ketogenic diet Pank2+/+ muscle (Fig. 9).

**Plasma analysis**

Plasma analysis showed an increase of cholesterol (Supplementary Fig. 2A) and triglycerides (Supplementary Fig. 2B) in mice on a
ketogenic diet. As expected, pantethine was able to reduce the levels of both (Supplementary Fig. 2A and B). A decrease of glucose levels was detected in mice fed a ketogenic diet and a ketogenic + pantethine diet (Supplementary Fig. 2C). Ketosis was observed in mice fed a ketogenic diet and was maintained during pantethine administration (Supplementary Fig. 2D).

Discussion

The Pank2<sup>−/−</sup> mouse model did not recapitulate the clinical and neuropathological features of the human condition (Kuo et al., 2005; Brunetti et al., 2012). Based on the role of co-enzyme A in several crucial metabolic pathways and considering the data obtained by a metabolomics approach in patients with PKAN, indicating the presence of impairment in lipid metabolism, we tested the hypothesis to challenge this mouse model with a diet containing high fat levels. Ketogenic diet consists of a low glucose and high lipid content, stimulating lipid use by mitochondrial beta-oxidation and ketone body production in the liver. Ketone bodies are high-energy-content compounds that can be used as an energy source by the brain, heart and skeletal muscle. We administered a low carbohydrate high-fat ketogenic diet to 2-month-old Pank2<sup>−/−</sup> and Pank2<sup>+/+</sup> mice and evaluated the clinical and biochemical phenotype.

We demonstrate here that the introduction of the ketogenic diet resulted in the onset of a severe phenotype in Pank2<sup>−/−</sup> mice characterized by motor dysfunctions, neurological impairment with feet-clasping, and exacerbated mitochondrial alterations, which were also present in the brain and PNSs of 12-month-old Pank2<sup>−/−</sup> mice on a standard diet (Brunetti et al., 2012). Moreover, this diet caused the premature death of Pank2<sup>−/−</sup> mice.

Pank2<sup>−/−</sup> mice fed a ketogenic diet did show the clinical signs present in patients with PKAN, namely more severe movement disorder and neurodegeneration. Importantly, these animals showed histological and immunohistochemical features of neurodegeneration, with cytoplasmic accumulation of abnormal, ubiquitinated proteins as observed in the brains of patients with PKAN (Kruer et al., 2011). However, the exact nature of the ubiquitinated proteins in our model remains to be elucidated. As observed in humans, cytoplasmic inclusions in Pank2<sup>−/−</sup> mice were negative for α-synuclein, confirming that PKAN neuropathological findings are different from other forms of neurodegeneration with brain iron accumulation.

We did not observe iron accumulation in Pank2<sup>−/−</sup> mice on a ketogenic diet, in contrast to that observed in human PKAN brains. We cannot exclude that iron levels could be below the detection level for the histological technique or that iron accumulates over a period of time beyond our observation. These aspects are still to be clarified and require further investigation.

The induction of a PKAN-like phenotype in Pank2<sup>−/−</sup> mice fed with a ketogenic diet, allowed us to have a model in which to test therapeutic compounds. Recently, in the Drosophila dPANK/fbl mutants it was shown that pantethine can work as a precursor of co-enzyme A, even in the presence of severely reduced levels of...
functional pantothenate kinase and that it was able to rescue brain degeneration, mitochondrial dysfunction and locomotor disabilities (Rana et al., 2010). By reasoning on these data we decided to administer pantethine to mice under ketogenic treatment and, as an internal control, to mice on a standard diet. In \textit{Pank2}\textsuperscript{-/-} mice on a ketogenic diet, we observed the rescue of the clinical phenotype including the movement disorder, the amelioration of the mitochondrial dysfunctions, and the extension of the lifespan as previously demonstrated in \textit{Drosophila} (Rana et al., 2010).

Treatment with pantethine dramatically improved both the histo-logical features of neurodegeneration and the ultrastructural mito-chondrial damage in \textit{Pank2}\textsuperscript{-/-} mice fed a ketogenic diet. We did not observe rescue of the azoospermic phenotype in mice. Fertility was not thoroughly investigated in patients with PKAN because of the severity of their clinical presentation and shortened lifespan. However, analysis of sperm samples in two affected individuals showed aberrant morphology and altered motility (Gregory and Hayflick, 2005).

We also demonstrated that pantetheine administration was able to rescue the mitochondrial phenotype in neurons derived from \textit{Pank2}\textsuperscript{-/-} mice on a standard diet, indicating that its effectiveness was not dependent on or influenced by the ketogenic treatment. It is known that pantetheine is rapidly converted into cysteamine and pantothenate by the vanin enzyme, also known as pantetheine hydrolase or pantetheinase (Kaskow et al., 2012). Although pantetheine is not able to cross the blood–brain barrier, Bousquet et al. (2010) demonstrated that cysteamine can cross the blood–brain barrier and in so doing can exert positive effects on the striatum and substantia nigra (Gibrat and Cicchetti, 2011). Cysteamine is able to enhance the expression of tyrosine hydroxylase protein and of the \textit{Nurr1} gene (now known as \textit{Nr4a2}), and to upregulate the expression of the Brain Derived Neurotrophic Factor (BDNF). These neuroprotective effects of cysteamine and of its dimer cystamine have been hypothesized to reflect the positive actions of this compound in Parkinson’s disease (Sun et al., 2010; Gibrat and Cicchetti, 2011) and Huntington’s disease (Borrell-Pages et al., 2006).

It has been observed that cysteamine and pantetheine in drinking water have beneficial effects in MPTP-induced mouse models of Parkinson’s disease (Cornille et al., 2010; Sun et al., 2010) and
would prevent neuronal degeneration in animal models of Parkinson’s disease (Stack et al., 2008; Gibrat and Cicchetti, 2011). Moreover, in animal models of Huntington’s disease, cysteamine exerts its neuroprotective effects by prolonging life span and decreasing motor symptoms (Dedeoglu et al., 2002; Karpuj et al., 2002).

In agreement with these data, we can hypothesize that the beneficial effects of pantethine in our model system were due to its conversion into pantothenate and cysteamine. In fact, pantethine is rapidly hydrolyzed to pantotenic acid and cysteamine as it could not be detected in plasma after oral administration (Wittwer et al., 1985). Cysteamine, the reduced form of cystamine (2-aminoethanethiol) is approved by the FDA for the treatment of cystinosis, a childhood disorder, which causes renal failure through cystine intracellular accumulation (Dohil et al., 2010) and in 2006 a small trial with this compound was initiated in patients with Huntington’s disease (Dohil et al., 2010).

However, cysteamine causes side effects (Corden et al., 1981) whereas pantethine has low toxicity (Knott et al., 1957; Schwartz and Bagdon, 1964) and might act as a neutral systemic carrier that would target cysteamine into the brain, avoiding toxicity and maximizing its efficacy.

The metabolism of cysteamine generates several intermediates including hypotaurine and taurine. In addition to being the major bile salt, in the form of taurocholate (Bouckenooghe et al., 2006), taurine crosses the blood–brain barrier and is involved in brain physiological activities such as inhibitory neurotransmission and long-term potentiation (Muramatsu et al., 1978; Pasantes-Morales et al., 1981). Recently, we observed a reduction of the bile acids tauro and glycol cholate (Leoni et al., 2012) and an alteration of lipid metabolism in plasma derived from patients with PKAN, likely because of co-enzyme A shortage causing, among others, dysfunctional fat assimilation.

Together with the data obtained in mice fed a high-fat diet, these observations suggest that it is possible to trace a parallel between patients with PKAN and Pank2^{-/-} mice under stressful conditions. Moreover, it is important to consider that environmental factors, such as food intake, together with genetic background could modulate the disease presentation by worsening or, on the contrary, stabilizing the progression of the symptoms. This could also explain the variability of the clinical presentation of the disease, with a spectrum of syndromes ranging from rapid to slowly progressive. Interestingly, we have analysed for the first time the muscle histology of a genetically defined patient with PKAN. The first neuromuscular examination was performed by Malandrini et al. (1995) in two adult cases of clinically-defined Hallerworden-Spatz disease, which showed the presence of subsarcolemmal myeloid structures, features characteristic of inflammatory myopathies.

In our study we demonstrated that the muscle of a 6-year-old patient with PKAN showed giant mitochondria with a mild alteration of the cristae and was histologically comparable with the muscle of Pank2^{-/-} mice fed a ketogenic diet. These alterations were rescued in mice after pantethine administration. This is a relevant observation when considering the option of pantethine administration to patients, as muscle analysis could represent the quantitative read-out to evaluate the effect of the compound with a minimal invasive procedure.

Taken together, these data strongly suggest that pantethine administration to patients with PKAN should be considered as a possible, safe and non-toxic therapeutic approach. Moreover, our data clearly demonstrate that an altered lipid metabolism, as a result of co-enzyme A shortage, could represent one of the underling causes of the disease. It is also possible, as demonstrated by the presence of mitochondrial alteration in the mouse model and in the muscle of a patient with PKAN, that mitochondria play a relevant role or could be a concurrent cause in the pathogenic mechanism of the disease. Interestingly, the presence of giant mitochondria in muscle, although with slight morphological differences, resembles the picture present in another human disorder caused by mutations in the CHKB gene and mainly characterized by muscular dystrophy and mental retardation (Quinlivan et al., 2013). CHKB encodes an enzyme catalysing the first step in de novo phosphatidylcholine synthesis (Mitsuhashi et al., 2013). CHKB and PANK2 are clearly part of different metabolic pathways but they converge on phospholipid biosynthesis. It is tempting to speculate that dysfunction of lipid metabolism is indeed the common culprit of the generation of mitochondrial muscle alteration observed in both disorders (Lamari et al., 2013).

We believe that the modulation of the diet composition in Pank2 knockout mice has generated a useful model system in which to test not only pantethine, but also additional compounds, which could be beneficial for patients.

Acknowledgements

We would like to thank Stefano D’Arrigo for the clinical data of the patient with PKAN. A special thank to Emilio Ciusani, Stefania Saccucci and Anil Rana for technical support.

Funding

The financial support of Telethon GGP11088 to V.T. is gratefully acknowledged. This work was supported by TIRCON project of the European Commission’s Seventh Framework Programme (FP7/2007-2013, HEALTH-F2-2011, grant agreement No. 277984).

Supplementary material

Supplementary material is available at Brain online.

References


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