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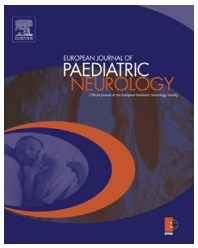

Original article

A family with paroxysmal nonkinesigenic dyskinesias (PNKD): Evidence of mitochondrial dysfunction

Daniele Ghezzi [a,1], *Carlotta Canavese* [b,1], *Gordana Kovacevic* [c], *Dragan Zamurovic* [c], *Chiara Barzaghi* [a], *Carlotta Giorgi* [d], *Giovanna Zorzi* [b], *Massimo Zeviani* [a], *Paolo Pinton* [d], *Barbara Garavaglia* [a], *Nardo Nardocci* [b,*]

[a] *Molecular Neurogenetics Unit, Fondazione IRCCS Istituto Neurologico "Carlo Besta", Milan, Italy*
[b] *Neuropediatrics Unit, Fondazione IRCCS Istituto Neurologico "Carlo Besta", Milan, Italy*
[c] *Department of Neurology, Institute for Mother and Child Health Care of Serbia, Serbia*
[d] *Dept of Morphology, Surgery and Experimental Medicine, Section of General Pathology, Interdisciplinary Center for the Study of Inflammation (ICSI), Laboratory for Technologies of Advanced Therapies (LTTA), University of Ferrara, Ferrara, Italy*

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ABSTRACT

Introduction: Paroxysmal nonkinesigenic dyskinesia (PNKD) is a rare movement disorder characterized by sudden attacks of involuntary movements. Familial PNKD is an autosomal dominant trait, caused by mutations in the myofibrillogenesis regulator 1 (*MR-1*) gene on chromosome 2q35. Three different mutations have been described; all of them reside in the N-terminal region common to isoforms L and S, that has been suggested to code for a mitochondrial targeting sequence, necessary for the correct sub-cellular localization of the protein into mitochondria.

Methods: We report on four patients of the same family, affected by PNKD. Skin fibroblasts were used to analysed oxygen consumption and to measure mitochondrial matrix calcium response after agonist stimulation. Mitotracker-based visualization was also used to assess fragmentation of the mitochondrial network.

Results: the paroxysmal movements were dystonic in two patients and dystonic/choreiform in the other ones; in three cases the symptoms started in one limb and then generalized, while in one case remained focal. Three had a very early onset, within the first two years of life. The frequency of episodes showed a great variability, ranging from 2 times a day to 3 times a year, while the duration of the attacks ranged from 2 min to 1,5 h, always with sudden onset and end and complete recover in between. All affected subjects harbored a heterozygous C to T substitution in *MR-1*, causing an Ala9Val amino acid change in the N-terminal region.

* *Corresponding author*. U.O. Neuropsichiatria Infantile, Dipartimento di Neuroscienze Pediatriche Istituto Fondazione IRCCS Carlo Besta, v Celoria 11 20133-Milano, Italy.
E-mail address: nardo.nardocci@istituto-besta.it (N. Nardocci).
[1] Equal contributors.
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A significant reduction of oxygen consumption and altered calcium homeostasis were found in mutant fibroblasts compared to controls, while no difference was detected in mitochondrial network.

Conclusions: The data on reduced oxygen consumption and altered calcium homeostasis obtained on mutant fibroblasts are the first evidences, in physiological conditions, of a mitochondrial dysfunction in PNKD.

1. Introduction

Paroxysmal nonkinesigenic dyskinesia (PNKD) is a rare movement disorder first described by Mount and Reback[1] in 1940 under the name "Familial paroxysmal choreoathetosis" and then reviewed by Demirkiran and Jankovic[2] in 1995. It is characterized by unilateral or bilateral attacks of involuntary movements, occurring spontaneously or precipitated by alcohol, coffee or tea, emotional stress or fatigue, but not by sudden movements or physical exertion.[3]

Attacks have never been associated with loss of consciousness or with seizures, and never reported to occur during sleep. The interictal neurological examination is usually normal as well as ictal and interictal Electroencephalography (EEG) and cerebral Magnetic Resonance Imaging (MRI). Age of onset is typically in childhood or early teens.

Familial PNKD is inherited in an autosomal dominant manner, with a high but incomplete penetrance of approximately 80%; mutations in the myofibrillogenesis regulator 1 (*MR-1*, or *PNKD*) gene on chromosome 2q35 are the only known genetic cause for PNKD,[4–7] albeit a second locus responsible for a familial form of PNKD was described on chromosome 2q31.[8] *MR-1* is transcribed into three alternatively spliced forms: long (MR-1L), medium (MR-1M) and small (MR-1S). Only three different mutations have been identified in *MR-1*; two missense mutations (Ala7Val, Ala9Val) have been found in several families with different ethnic origin, whereas a third missense change (Ala33Pro) was described in a single Italian family. All the three mutations described until now in *MR-1* reside in the N-terminal region common to isoforms L and S, that has been suggested to code for a mitochondrial targeting sequence (MTS), necessary for the correct sub-cellular localization of the protein into mitochondria.[7]

We report on four subjects from the same family, affected by PNKD and mutated in *MR-1*. Functional studies performed in mutated cultured fibroblasts suggest a dysfunction of mitochondria.

2. Methods

We retrospectively reviewed clinical data of four patients over three generations of the same family (Fig. 1, pedigree).

All patients were clinically evaluated at the Department of Neurology, Institute for Mother and Child Health Care of Serbia. Three of them (III:1, II; 2 and II; 3) underwent EEG and cerebral MRI. After obtaining informed consent, the DNA of all patients were analyzed for *MR-1* gene mutations in the Molecular Neurogenetics Unit, Institute of Neurology "Besta".

Genomic DNA was extracted from blood by standard methods. The twelve exons and intron–exon junctions of the three MR-1 isoforms were polymerase chain reaction amplified using suitable primers[4] and analyzed by Sanger sequencing in proband/subject III-1. The exon1 was amplified and sequenced in all available family members.

2.1. Cell culture and biochemical assays

Skin fibroblasts from proband (III:1) and control subjects were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 mg/ml streptomycin, 100 units/ml penicillin, and 1 mM sodium pyruvate at 37 °C in a humidified incubator containing 5% CO_2. Oxygen consumption rate (OCR), maximum respiration rate (MRR), respiratory control ratio (RCR) and spare respiratory capacity (SRC) were measured with an XF96 Extracellular Flux Analyzer (Seahorse Bioscience).[9] Data were expressed as pmol of O_2 per minute and normalized by cell number measured by the CyQUANT Cell proliferation kit (Invitrogen™).

For Ca^{2+} measurements, fibroblasts were transfected with a mitochondrial targeted aequorin and mitochondrial Ca^{2+} measurements were carried out exactly as previously described.[10] For mitochondrial network visualization, the mitochondrial fluorescent dye, MitoTracker®Red CMXRos

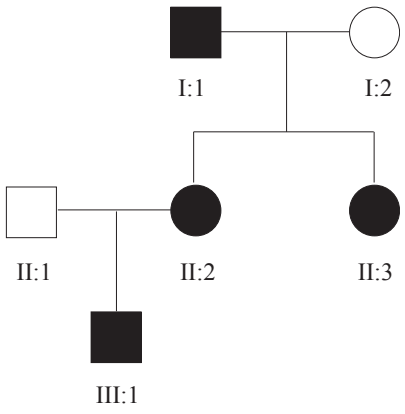

Fig. 1 – Pedigree of the family. Black symbols indicate affected subjects, all heterozygous for the c.26C > T/ p.Ala9Val mutation.

(Invitrogen) was added into the culture media at final concentrations of 50 nM. The cells were incubated under normal culture conditions for 30 min, and then visualized by fluorescence microscopy (Nikon-CARV2 system).

3. Results

3.1. Clinical features

The proband (III:1) is 7-year old boy who presented, since the second year of life, with attacks of dystonic movements of the arms, spreading to the legs, sometimes associated with jaw stiffness and mouth dystonia. The attacks lasted from 5 min to 1 h and their frequency ranged from 1 to 10 per month; no precipitating factor has been noticed. His personal history was unremarkable; interictal neurological examination, laboratory and metabolic investigations and brain MRI were normal. Interictal EEG showed normal background activity and rare sharp-waves over right centro-temporal region. He was initially treated with carbamazepine but his involuntary movements were getting worse, with frequency increasing to 1–3 per day. Further treatment with clobazam and clonazepam caused only temporary improvement.

The mother (II:2) had the first attack of choreiform and dystonic movements in her arms when she was one year old. During the first 18–20 years of her life she had only mild and rare dystonic episodes. Her condition was getting worse after the child's delivery. Since then, she has had attacks of generalized choreoatetosis affecting both upper and lower limbs, which were accompanied by stiffness of the jaw, dystonia of the mouth, and a feeling of "generalized stiffness". These attacks were long lasting (1–1.5 h), occurred one or two times a day and were triggered by stress and caffeine intake. Her personal history, interictal neurological examination, laboratory tests and EEG were unremarkable. Treatment with antiepileptic drugs as Phenobarbiton, Carbamazepine, Clobazam, Midazolam, and Levetiracetam was unsuccessful.

The aunt of the proband (II:3) presented, since the first year of life, with attacks of dystonic and choreiform movements involving both hands and feet, not interfering with normal walking, associated with muscle stiffness and difficulty in speaking. The attacks lasted usually 2–10 min, their frequency ranged from 1 per month to 3 for years and they were triggered by stress and caffeine intake. Her personal history and neurological examination were unremarkable. Her 7-year old child has not similar symptoms. Medical treatment with Phenobarbiton and Carbamazepine was unsuccessful.

The grandfather of the proband (I:1) noticed the first attack of choreoatetotic movements in his hands, associated with difficulty in speaking, at age of 15 years. Compared with the other affected family members, he had the mildest episodes, which occurred 1–2 times per months and lasted 5–10 min. Caffeine intake, stress and alcohol consumption triggered attacks. He had never visited doctor because of this problem. He didn't require any medical treatment. His second daughter and her 3 children have not similar episodes.

3.2. Genetic and functional studies

By sequencing of the *MR-1* gene, we found that all affected subjects of the family harbored a heterozygous C to T substitution (c.26C > T; NM_015488.4) resulting in an amino acid change (p.Ala9Val) in the N-terminal region common to isoforms MR-1L and MR-1S.Since MR-1S and MR-1L have been found to have a mitochondrial localization, we tested if the presence of the A9V mutation has an impact on mitochondrial functions. We used fibroblasts of the proband (III:1) that expressed MR-1S isoform. We couldn't evaluate MR-1L isoform since it is expressed only in brain.[4]

We analysed the oxygen consumption, which depends upon and reflects the cumulative proficiency of the whole set of mitochondrial respiratory chain (MRC) complexes. We found a significant reduction of different parameters in the proband's cells compared to control fibroblasts: the maximal respiration rate (MRR), indicating reduced electron flow through the respiratory chain, and the respiratory control ratio (RCR), an indicator of the bioenergetic reserve. There was also a slight, but significant, decrease of the spare respiratory capacity (SRC), an index of mitochondrial coupling between the MRC and the ATP synthase (Fig. 2A-D).

Then, mitochondrial matrix calcium (Ca^{2+}) measurements were carried out using aequorin-based recombinant probes.[10] We decided to investigate the mitochondrial Ca^{2+} response after agonist stimulation as a highly sensitive readout of mitochondrial state. Indeed, mitochondrial alterations cause defects in Ca^{2+} uptake by the organelle.[11] Fibroblasts were exposed to ATP (100 μM), causing the generation of inositol 1,4,5 trisphosphate (IP3) and the consequent release of Ca^{2+} from the endoplasmic reticulum to mitochondria.[12] In patient's fibroblasts, the mitochondrial Ca^{2+} concentration increase evoked by the agonist was significantly greater compared to control fibroblasts (163.70 ± 14.51 μM vs. 116.7 ± 16.54 μM), indicating an impairment in mitochondrial homeostasis (Fig. 2E). Finally, Mitotracker-based visualization was used to assess fragmentation of the mitochondrial network, which is a well-documented alteration related to reduced fusion of mitochondrial membranes and is typically observed in cells with severe MRC impairment or in pre-apoptotic conditions.[13] No evident difference was detected in mutant fibroblasts compared to control cells (not shown).

4. Discussion

Involuntary movements typical of PNKD include dystonic posturing with choreic, ballistic or athetotic movements: 80% of genetically proven cases were found to have a combination of dystonia and chorea, 12% had dystonia only. Movements usually begin on one side and tend to spread or even generalize[3,14]; age of onset is typically in childhood or early teens, with a mean age of 8 years.[14] In our family involuntary movements were dystonic in two patients and dystonic associated with choreiform movements in the other two; in three cases started in one limb and then tended to generalize, while in one case remained focal. Three of our patients had a

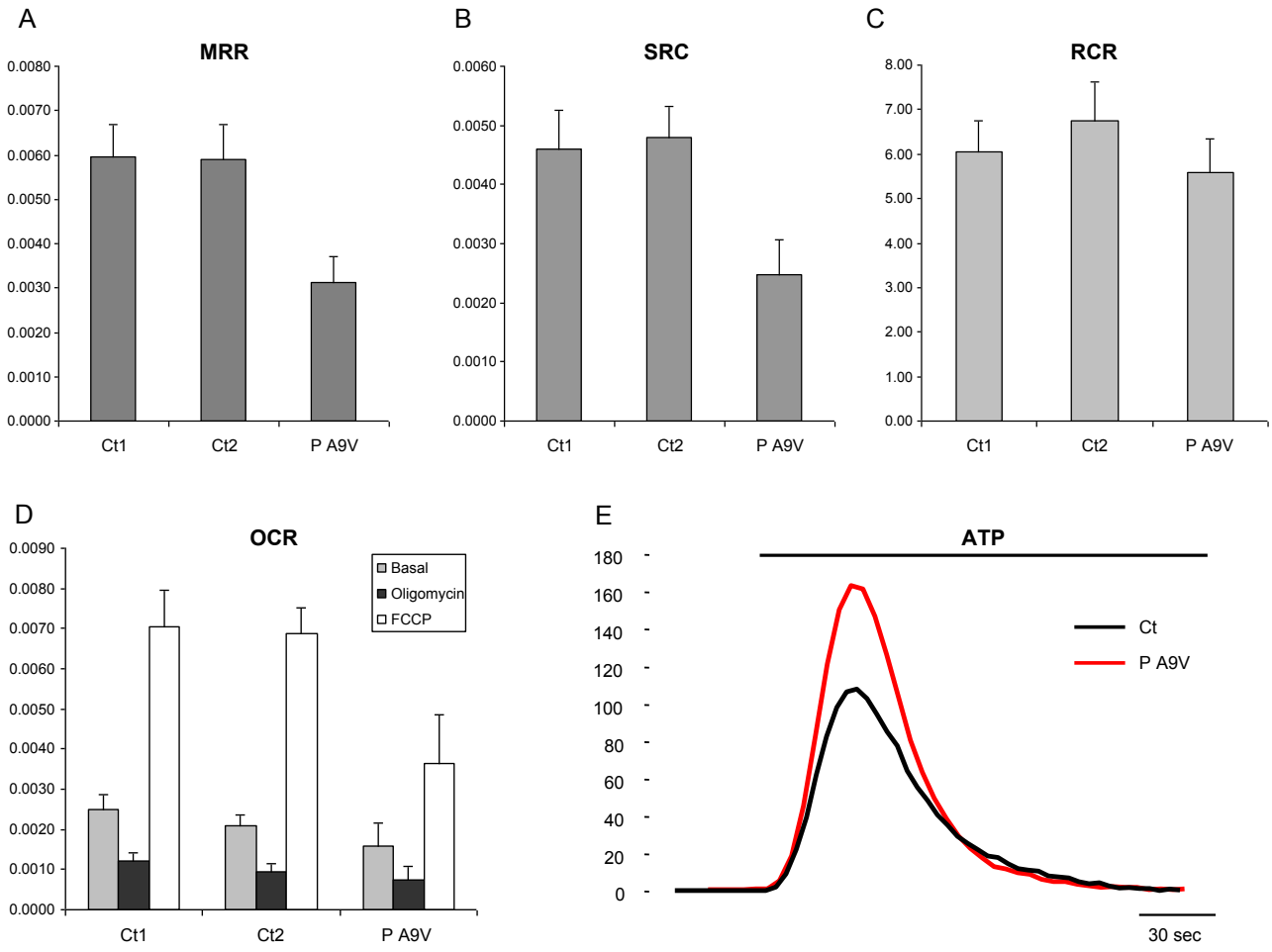

Fig. 2 – Oxygen consumption analysis and calcium response in patient's and control fibroblasts. Histograms showing maximum respiration rate (MRR, panel A), respiratory control ratio (RCR, panel B), spare respiratory capacity (SRC, panel C), and oxygen consumption rate (OCR, panel D) measured in basal condition and after addition of oligomicyn and FCCP. Fibroblasts from two age- and sex-matched controls (Ct1, Ct2) and from patient III-1 (P A9V) were analysed. MRR, RCR and OCR values are expressed as pmol of O_2 per minute and normalized by cell number; SRC is an adimensional ratio. The graphs represent The mean values from 2 independent experiments, each with 6–8 replicates. Two-tailed Student's t-tests P A9V vs Ct (Ct1 + Ct2): MRR $p = 1.1 \times 10^{-14}$; SRC $p = 2{,}9 \times 10^{-12}$; RCR $p = 0.0011$. Panel E: Mitochondrial Ca^{2+} response after agonist stimulation (ATP 100 μM) in control (Ct) and patient III-1 (P A9V) fibroblasts, measured using a recombinant aequrin probe.

very early onset, within the first two years of life, whereas the fourth had the first symptoms during adolescence.

Attacks may begin with premonitory symptoms (41% of genetically proven cases) such as a sensation of tightness in one limb, involuntary movements of the mouth or anxiety. Attacks usually last minutes to hours and rarely occur more than once per day, but frequency, duration and severity, as well as combinations of symptoms in terms of movement type and location, vary broadly within and among families.[3,15] This phenotypic variability is well expressed in our family, especially in terms of frequency of the episodes, ranging from 2 times a day to 3 times a year. Interestingly patient II-2, who had the highest frequency of attacks, had a worsening after a delivery. There are no reports in literature about relation between pregnancy or delivery and PNKD, whereas worsening during menstruation or ovulation period has been described.[14]

In all our cases there was an oro-mandibular muscles' involvement with inability to open the mouth and to speak, associated with the movement disorder. As described in literature, response to pharmacological treatment was poor; beside clonazepam or diazepam, that are sometimes effective, there are reports on efficacy of gabapentin and levetiracetam.[16,17] However, the latter was tried in patient II-2 without any clinical improvement.

All known PNKD mutations, including the change found in the present family, affect the N-terminal region, common to isoforms MR-1L and MR-1S, coding for an MTS. We previously demonstrated that these two isoforms are located into mitochondria, suggesting that mitochondria could have a role in the pathogenesis of PNKD.[7] The data on reduced oxygen consumption and altered calcium homeostatsis obtained in this study on mutant fibroblasts are the first evidences, in

physiological conditions, of a mitochondrial dysfunction in PNKD. However, the absence of clear alterations in the mitochondrial network indicates that only some mitochondrial functions are altered, at least for Ala9Val mutation and in cells like fibroblasts expressing MR-1S, but not MR-1L. Moreover the unexpected increase in calcium uptake, rather than the decrease usually present in other mitochondrial dysfunction, requires further studies to better understand the functional link between mutations in MR1 and cellular calcium handling.

Conflicting findings on sub-cellular localization of the different MR-1 isoforms have been reported,[4,7,18] in particular regarding MR-1L; however, there is a general consensus about an N-terminal cleavage leading to the mature protein. Since all the known mutations in MR-1 are located in this region (probably an MTS), it has been hypothesized that either the maturation process could be altered or the mutant MTS has itself a deleterious role, for instance a toxic effect.[7,18] These damaging consequences could convey the effects of other precipitating stimuli, resulting in variable penetrance and clinical seriousness. Moreover, they are probably cell-type specific. While MR-1S has a ubiquitous distribution, the selective expression of MR-1L in the brain, hampered the evaluation of the effect of mutated MR-1L isoform on patients' cells; being PNKD a neurological disorder, this is clearly a limit to fully understand the role of mitochondria in PNKD. The use of fibroblast-derived induced pluripotent stem cells differentiated into neurons could be useful in the future to confirm and better define the impairment of mitochondrial function in PNKD, to elucidate the role of MR-1L in the disease, and eventually to take advantage of this knowledge for the development of new therapeutic approaches.

Conflict of interest

The authors thanks the “Fondazione Mariani” for the support.

The authors have no potential or actual conflict of interest nor financial disclosures.

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