Abstract

We report a newborn with exaggerated startle reactions and stiffness of neonatal onset, the prototypical signs of hyperekplexia. Startle and flexor spasms, leading to apnoea, did not respond to treatment with clonazepam but did partially to sodium valproate. Molecular analysis of GLRA1 revealed no mutations. The incidental finding of hypouricemia led to a work-up for molybdenum cofactor (MoCo) deficiency; the diagnosis was confirmed by the altered urine chemistries, including elevated urine S-sulphocysteine. Despite persistence of the spasms, clinical or electrographic seizures were never detected before the infant died at age 1 month. In this patient, the concurrence of hyperekplexia and MoCo deficiency was suggestive of impaired gephyrin function. GPH mutational analysis, however, showed no abnormalities. The patient was eventually found to harbour a novel c.1064T > C mutation in exon 8 of the MOCS1 gene. Despite extensive sequence analysis of the gene, the second causative mutation of this recessive trait still awaits identification. The patient was eventually found to harbour a novel c.1064T > C mutation in exon 8 of the MOCS1 gene. Despite extensive sequence analysis of the gene, the second causative mutation of this recessive trait still awaits identification. MoCo deficiency should be considered in the differential diagnosis of neonatal hyperekplexia, particularly in the instances of refractoriness to clonazepam, an early demise in infancy or the evidence of no mutations in the GLRA1 gene.

Key words
Startle · newborn · molybdenum · gephyrin

Introduction

Molybdenum cofactor (MoCo) deficiency (MIM# 252150) is a neonatal-onset neurological disorder with autosomal recessive inheritance and rapid, severe neurological deterioration. The usual phenotype consists of untreatable neonatal seizures and profound retardation in association with the biochemical findings of hypouricemia, elevated xanthine in plasma and urine and elevated urine sulphites, particularly S-sulphocysteine. Most patients do not survive the neonatal period. The deficiency of MoCo results in loss of activity of the molybdoenzymes sulphite oxidase, xanthine dehydrogenase and aldehyde oxidase, the neurological damage being attributed primarily to sulphite oxidase deficiency. At least four different proteins are involved in the biosynthetic pathway of MoCo. Mutations in three of them, MOCS1, MOCS2 and GPH, have been identified in patients with MoCo deficiency [29,30]. MOCS1, which encodes two peptides (MOCS1A and MOCS1B) that convert a guanine derivative into protein Z, and MOCS2, encoding the two subunits of molybdopterin synthase (MOCS2A and MOCS2B), are bicistronic genes. Each of them can produce two proteins either from different mRNAs generated by alternative splicing or by independent translation of a bicistronic mRNA [30]. GPH encodes gephyrin, a postsynaptic scaffolding protein that is also required for the insertion of molybdenum during cofactor assembly. Gephyrin is involved in clustering the inhibitory glycine and GABA$_A$ receptors at the postsynaptic membrane [6,13].
Abnormal, excessive startle to unexpected stimuli is the key feature of hyperekplexia (MIM# 149400, startle disease, STHE, Kok disease) [14,16,36]. The neonatal – or major – variant of the disorder is characterised by accompanying episodes of acute generalised flexion hypertonia, sometimes leading into apnoea, and a strong head-retraction reflex upon nose tapping [1,32]. In addition, many affected infants display continuous muscle rigidity, referred to in the past as the stiff baby syndrome [18, 31]. Several reports have alerted to the potentially fatal outcome of hyperekplexia with neonatal onset [17,20,21,37]. Hyperekplexia is often a hereditary condition, more commonly autosomal dominant with complete penetrance and variable expression, although cases with incomplete penetrance [15] or with autosomal recessive inheritance [24] have been reported. Point mutations in the GLRA1 gene, encoding the glycine receptor alpha1 subunit, have been found in familial hyperekplexia [33,34]. A transient hyperekplexia phenotype has been associated with compound heterozygote mutations in the gene GLRB, encoding the beta subunit of the glycine receptor [25]. The glycine transporter subtype 2, a presynaptic transporter located in glycinergic interneurons, has also been considered a candidate hyperekplexia gene, since GlyT2-deficient mice display lethal neuromotor signs that mimic human startle disease [8]. In all these instances, as a result of altered glycine receptor function, the balance between excitatory and inhibitory transmission in the brainstem and spinal cord is lost toward neuronal hyperexcitability. The molecular basis in a substantial number of cases of sporadic and hereditary hyperekplexia, however, remains unknown.

We report the case of a neonate who presented at birth with the association of the classical hyperekplexia phenotype and the characteristic biochemical findings of MoCo deficiency. The unique phenotype of this patient hinted at a disturbed gephyrin function; however, while the analysis of GPH coding sequence revealed no mutations, he was found to harbour a novel mutation in exon 8 of the MOCS1 gene.

Patient and Methods

Case report

This Caucasian male infant was born at 34 weeks gestation to healthy, non-consanguineous parents. The mother was a 37-year-old gravida 3 para 2, who had a normal pregnancy until week 32 when she was admitted to the hospital because of premature labour, which was controlled with tocolysis. Foetal movements had been felt as normal and amniocentesis had yielded a normal karyotype. Foetal parenchymal maturation with steroids was started. A foetal ultrasonogram at 33 weeks detected moderate polyhydramnios and suggested macrosomy. At 34 weeks, amniorrhesis was followed by an uncomplicated vaginal delivery. Birth weight was 2700 g (90th percentile), length 45 cm (50th percentile) and head circumference 34 cm (97th percentile). Apgar scores at 1 and 5 min were 5 and 6, respectively. Immediately after birth the infant developed generalised jerks, rigidity and apnoeic spells, labelled initially as seizures and treated with phenobarbital and midazolam. He was intubated and transferred to the neonatal ICU. Family history was negative for symptoms associated with hyperekplexia or other neurological disorders.

During the following days the patient remained alert but severely hypoactive and hypertonic, with flexed forearms and legs and generalised hyperreflexia. Stiff stimuli or handling triggered generalised tonic spasms with apnoea. Tapping the nasal bridge or tip produced a generalised startle with prominent head retraction that showed no habituation. Several EEG recordings, both ictal and interictal, were normal. A cranial ultrasonogram was normal. A CSF analysis revealed normal glucose, proteins and cell counts. Microbiological investigations were negative. EKG monitoring did not reveal a heart block. Initial metabolic studies, including blood glucose, ammonia, acid-base status, lactate and pyruvate, revealed no abnormalities.

On day 3, phenobarbital was discontinued and clonazepam was titrated up to 0.25 mg/kg/day, with a mild improvement of hypertonia but persistence of startle reactions, both spontaneous and stimulus-induced. An occasional, severe attack, which prevented even mechanical ventilation, was aborted by flexing the patient toward the trunk, as described by Vigevano et al. [37]. On day 8, a further attempt to control the symptoms by adding sodium valproate, up to 30 mg/kg/day, resulted in a reduction of the number and intensity of the tonic spasms and startle reactions and the emergence of some spontaneous movements, but the drug was discontinued later on, as the general status of the patient deteriorated. He developed a left inguinal hernia, that was operated on day 17. No dysmorphic features were ever noted. The patient’s clinical course was complicated by repeated bouts of atelectasia and pneumonia, which precluded weaning from mechanical ventilation, and by progressive deterioration of renal glomerular and tubular function, with generalised oedema, proteinuria and hypoalbuminemia. A renal ultrasonogram was normal. Towards the end of the first month he was comatose, with profound hypotonia, absent spontaneous movements and very occasional episodes of hypertonia. Clinical or electrographic seizures were never documented. From day 20 onwards, the patient developed multiorgan failure that did not respond to intensive care and the patient died at age 40 days. Necropsy was not available.

Biochemical studies

Serum and urine urate were low at 23.8 μmol/L (normal 136.8 – 279.6) and 0.0095 mmol/day (normal 1.5 ± 0.3), respectively. Urine sulphite test was positive. Urine amino acid analysis showed increased taurine (676 μmol/mol creatinine; normal 8 – 226) and a peak compatible with sulphocysteine. Liquid chromatography for urine purine and pyrimidine profile revealed elevation of xanthine (429.1 mmol/mol creatinine; normal <68) and normal hypoxanthine (115 mmol/mol creatinine; normal <62). These findings were consistent with the biochemical profile of molybdenum cofactor (MoCo) deficiency. Mass spectrometric analysis verified and quantitated S-sulphocysteine: 579 μmol/g creatinine; normal <100 μmol/g creatinine (Dr. R. Stevens, Duke University, Durham, NC) and thus confirmed the diagnosis of MoCo deficiency. Serum amino acids, lactate and pyruvate and urine organic acids were normal.

Genetic studies

Genomic DNA isolation

Genomic DNA from the patient and his parents was isolated from venous blood using the QIAamp DNA Blood Midi Kit (Qiagen, Hil-
den, Germany). The samples were drawn in accordance with the Helsinki declaration.

Mutation analysis

The complete coding region and all the splice sites of the GPH (Gephyrin, MIM 603930), MOCS1 (Molybdenum Cofactor Synthesis 1, MIM 603707) and MOCS2 (Molybdenum Cofactor Synthesis 2, MIM 603708) genes were PCR-amplified from the patient’s genomic DNA. For MOCS1, 1859 bp preceding the MOCS1A initiation codon and 843 bp following the MOCS1B stop codon were also amplified in four segments. The PCR conditions used were the following: 50 ng genomic DNA, 1 U AmpliTaq Gold polymerase (Applied Biosystems, Branchburg, NJ, USA), 1 × recommended buffer, 1.5 mM MgCl2, 200 μM dNTPs, 10 pmol of both the forward and reverse primers in a final volume of 50 μL. The amplification protocol was 94°C for 10 min; 94°C for 30 s, 51 – 60°C for 45 s and 72°C for 30 sec, for 35 cycles; and 72°C for 2 min. For GLRA1 (Glycine Receptor Alpha 1 subunit, MIM 138491) analysis, genomic DNA was amplified by PCR with the following parameters: 95°C for 5 min, 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s and a final extension for 5 min at 72°C. The primer sequences used in the amplification of MOCS1, the PCR products length, and the particular PCR conditions used for some of the fragments are indicated in Table 1. Some of the primer sequences are according to Reiss et al. [28], whereas the rest were designed de novo to include longer intronic sequences. The primers for GPH and MOCS2 are available from the authors upon request. Most of the primer pairs were designed from genomic sequences using the Primer3 program (frodo.wi.genome.mit.edu/cgi-bin/primer3/primer3_www.cgi). The GLRA1 primers are according to Shiang et al. [34]. The PCR fragments were column-purified using the GFX™ PCR DNA and Gel Band purification Kit (Amersham Biosciences, Little Chalfont, UK) or the QiAquick PCR purification kit (Qiagen, Hilden, Germany). The samples were drawn in accordance with the Helsinki declaration.

Table 1 Primers for PCR amplification of the MOCS1 gene exons and flanking intron sequences, product sizes, and PCR reaction conditions

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer sequence (5’→3’)</th>
<th>Product length (bp)</th>
<th>Annealing temperature (°C)</th>
<th>DMSO %</th>
</tr>
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<tr>
<td>1a</td>
<td>F – gacctgtgctctgcctatt</td>
<td>339</td>
<td>51</td>
<td>yes</td>
</tr>
<tr>
<td>1bcd</td>
<td>F – gctgtaacttaaaggggt-</td>
<td>401</td>
<td>52</td>
<td>no</td>
</tr>
<tr>
<td>1cd</td>
<td>...</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>F – tgagggagttgagcag-</td>
<td>318</td>
<td>57</td>
<td>no</td>
</tr>
<tr>
<td>3</td>
<td>F – gagagctcctttccatc-</td>
<td>388</td>
<td>55</td>
<td>no</td>
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<tr>
<td>4</td>
<td>F – tttgcaatctcagacta-</td>
<td>209</td>
<td>55</td>
<td>no</td>
</tr>
<tr>
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<td>303</td>
<td>55</td>
<td>no</td>
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<tr>
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<tr>
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<td>55</td>
<td>no</td>
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<td>292</td>
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<td>no</td>
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<td>F – cccaggtgtctctgcc-</td>
<td>246</td>
<td>60</td>
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<td>330</td>
<td>58</td>
<td>no</td>
</tr>
<tr>
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<td>F – attttgtcttccccatct-</td>
<td>760</td>
<td>57</td>
<td>no</td>
</tr>
<tr>
<td>10c</td>
<td>F – agatccacagcattgc-</td>
<td>342</td>
<td>55</td>
<td>no</td>
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<tr>
<td>Prom-A</td>
<td>F – cgccccctcgtacacct-</td>
<td>875</td>
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<tr>
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<td>558</td>
<td>55</td>
<td>no</td>
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<tr>
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<td>577</td>
<td>55</td>
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<tr>
<td>3’-UTR</td>
<td>F – gacatgaggtctggg-</td>
<td>797</td>
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</table>

Results

Complete sequencing of the GPH, MOCS1, MOCS2 and GLRA1 coding sequences, all exon-intron boundaries and part of the 5′- and 3′-untranslated regions revealed a novel putative disease-causing mutation in exon 8 of the MOCS1 gene of the patient (Fig. 1).
This exon belongs to the 5′ open reading frame (MOCS1A) of the gene, which produces a bicistronic mRNA with the potential to produce two proteins (MOCS1A and MOCS1B). The mutation, inherited from the father, is a heterozygous T to C transition at position 1064 of the cDNA starting from the A of the initiation codon (c.1064T > C), causing an Ile to Thr amino acid substitution (p.Ile355Thr). The putative promoter region of the MOCS1 gene and the 3′-untranslated region were also sequenced in the patient, but no other mutation was identified in this gene or in any other gene included in this study.

Discussion

The patient we report expands the clinical phenotype of molybdenum cofactor deficiency by including hyperekplexia as a possible presenting sign.

Hyperekplexia, usually a hereditary condition, is an exaggeration of the normal startle reaction, with brief, superimposed attacks of hypertonia. Marked stiffness is a common, additional feature in affected infants with the major form of the disease [1,36]. In familial cases, the clinical phenotype is variable and can be limited to occasional excessive startle and/or nocturnal myoclonus. Hyperekplexia can also occur in adults as a consequence of acquired lesions involving the rhomboencephalic reticular formation in the lower brainstem, most probably at the level of the nucleus reticularis pontis caudalis [3,19]. Our patient, despite the preterm labour, did not show signs suggestive of intrapartum asphyxia or neonatal hypoxic-ischemic encephalopathy and neuroimaging did not reveal abnormalities. The patient was alert and seizures were not present either clinically or on the serial EEG’s. Still, the patient needed immediate ventilation because slight tactile or auditory stimuli elicited episodes of generalised hypertonia leading to apnoea and showed stiffness between these episodes. Presumably, as a consequence of the former, he also developed an inguinal hernia. As in some previous reports, we found the manoeuvre of forcefully flexing head and legs toward the trunk to be life-saving. The preceding clinical findings fit clearly the ones previously described in cases of neonatal hyperekplexia [22]. At variance with many reports on neonatal hyperekplexia, the clinical improvement after starting clonazepam was very limited in our patient. A more notable improvement of the neurological symptoms was seen when sodium valproate was added, similarly to a single case report with adolescent-onset hyperekplexia [4]. Both clonazepam and sodium valproate are GABAergic drugs and the efficacy in ameliorating pathological startle has been taken as indirect evidence of reduced GABA availability underlying deficient inhibitory transmission in hyperekplexia. Along this line, low CSF GABA has been found in several patients with hyperekplexia [2,5,35]. However, the only molecular defects identified so far in hereditary hyperekplexia are mutations in the genes encoding the alpha and beta subunits of the inhibitory glycine receptor [25,33]. The most common of them, mutations in GLRA1 gene, were not found in our patient. It is, in fact, estimated that some 60% of the sporadic hyperekplexia cases are not linked to GLRA1 or GLRB mutations [26].

The diagnosis of molybdenum cofactor deficiency was unexpected in our patient. Although hypertonia, dystonia or myoclonus have been occasionally reported in MoCo deficiency [9], exaggerated startle and stiffness, to the degree seen in hyperekplexia, are not among the previously reported clinical signs in this condition. Moreover, the typical clinical sign of tonic-clonic seizures was not present; neither were other typical signs, such as dysmorphic facies, feeding troubles or lens subluxation [11]. Consequently, the diagnostic suspicion was not raised until low serum uric acid was found during the second week of life. The
progressive renal failure of our patient has not been noted in previous patients with MoCo deficiency. Why MoCo deficiency should produce exaggerated startle is not obvious. It is conceivable that sulphite oxidase deficiency could mimic primary hyperekplexia because of a severe neuronal loss at either the cortical or brainstem level, causing deranged inhibitory circuitry, similarly to the proposed pathogenesis in the classical, seizure-prone phenotype. Unfortunately, neurochemical or pathological data to prove or disprove this hypothesis are lacking and neuro-radiological findings were limited to cranial ultrasonograms in our patient, given his critical condition since birth.

The combination of hyperekplexia and MoCo deficiency strongly suggested a disturbance in gephyrin function. Gephyrin is a key protein in the organisation of inhibitory postsynaptic receptors in human CNS. Gephyrin knock-out mice display both deficient MoCo biosynthesis and an abnormal motor behaviour reminiscent of human hyperekplexia [7]. A patient with MoCo deficiency, but no clinical signs suggesting hyperekplexia, was found to harbour a deletion of exons 2 and 3 in the GPH gene [29]. Despite the negative mutational analysis in our patient, GPH still appears as an adequate candidate gene for hereditary forms of hyperekplexia not linked to GLRA1 or GLRB mutations. Recently, in a series of 32 familial and sporadic cases of hyperekplexia, Rees et al. detected a single case with novel mutation causing an N10Y substitution at the extreme N-terminus of gephyrin. The patient developed a partially reversible hyperekplexia phenotype with no associated signs of altered molybdenum activity. However, functional analysis of this mutation showed that it did not disrupt targeting or clustering of glycine receptors [26].

Mutations in the MOCS1 and MOCS2 genes have been identified in patients with MoCo deficiency [30], whereas mutations in the GLRA1 gene are responsible for familial hyperekplexia [33,34]. An extensive mutational analysis of these three genes in our patient allowed the identification of a previously undescribed mutation in MOCS1, p.Ile355Thr. Several facts strongly suggest that this change is indeed a disease-causing mutation. First, it was not found in a screening of 150 control chromosomes from healthy Spanish individuals, nor in any public or private SNP database (Applera Genomics – www.celera.com, NCBI – www.ncbi.nlm.nih.gov, the SNP Consortium – snp.cshl.org and ENSEMBL – www.ensembl.org) indicating that it is not a neutral polymorphism present in the general population. Second, amino acid Ile355 is a conserved residue in the MOC51 protein of Homo sapiens (human), Mus musculus (mouse), Bos taurus (cow), Orcytolagus cuniculus (rabbit), Gallus gallus (chicken), Drosophila melanogaster (fruit fly), Caenorhabditis elegans (nematode), Monodelphis domestica (South American opossum) and many plant and bacteria species, indicating functional/structural relevance [10,27]. And third, this substitution causes a change from a non-polar to a polar amino acid residue. Furthermore, when the secondary structures for the normal and mutant proteins are predicted using the NNPREDICT software [12], substantial modifications are clearly shown, as the mutation disrupts a predicted α helix. However, further functional characterisation of the mutant allele will be needed to confirm these assumptions. Although we have sequenced all the coding region of the MOCS1 gene, the splice sites, the putative promoter and the 3′-untranslated region, including the polyadenylation signal, only one of two expected recessive disease alleles was identified in the patient, merely suggesting MOCS1A deficiency. It is possible that mutations in introns or distant regulatory regions affecting the transcript levels, or gross heterozygous genomic rearrangements, have remained undetected. Unfortunately, the limited availability of genomic DNA and RNA from the patient and his parents makes it difficult to perform Southern or Northern blot analysis. The mutation identified in our patient adds to the 16 changes previously reported by others in the MOCS1 gene in patients with MoCo deficiency, the majority in the MOCS1A ORF. Interestingly, molecular alterations in MOCS1A account for approximately 50% of all MoCo deficiency cases [30], but had never been associated with the hyperekplexia phenotype.

Genetic heterogeneity may account for the diverse clinical course in hyperekplexia. While some neonatal cases are described as relatively benign and reversible and show clear improvement when clonazepam treatment is started, other follow a relentlessly neurological deterioration or die suddenly in infancy. It is possible that other drugs may prove to be effective in these cases; propofol has been shown to potentiate glycine receptor responses in Xenopus oocytes and transgenic mice carrying “hyperekplexic” mutations in GLRA1 receptors [23].

Whatever its genetic basis, neonatal hyperekplexia can be a life-threatening condition. MoCo deficiency should be considered in the differential diagnosis of a neonate presenting with excessive startle and a poor response to standard treatment.

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