Case Report

Spontaneous Spongiform Brainstem Degeneration in a Young Mouse Lemur (*Microcebus murinus*) with Conspicuous Behavioral, Motor, Growth, and Ocular Pathologies

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Here we report a case of severe growth retardation and neurologic abnormalities in a female gray mouse lemur (*Microcebus murinus*), a small NHP species for which the genomic sequence recently became available. The female lemur we present here died on postnatal day 125. This lemur had impaired development of motor skills and showed severe ataxia and tremors. In addition, hearing seemed normal whereas ophthalmic examination revealed incipient bilateral cataracts, abnormal pigmentation in the lens of the left eye, and a missing optokinetic nystagmus, which indicated impaired vision. Most prominently, the lemur showed severe growth retardation. Necropsy revealed maldevelopment of the left reproductive organs and unilateral dilation of the right lateral ventricle, which was confirmed on brain MRI. Brain histology further revealed large, bilateral areas of vacuolation within the brainstem, but immunohistochemistry indicated no sign of pathologic prion protein deposition. Full genomic sequencing of the lemur revealed a probably pathologic mutation in *LARGE2* of the *LARGE* gene family, which has been associated with congenital muscular dystrophies. However, potentially functional mutations in other genes were also present. The observed behavioral and motor signs in the presented animal might have been linked to spongiform degeneration and resulting brainstem dysfunction and progressive muscle weakness. The macroscopic developmental abnormalities and ophthalmic findings might be genetic in origin and linked to the mutation in *LARGE2*.

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The nocturnal and omnivorous mouse lemurs are the world's smallest primates. Therefore, they can efficiently be maintained and bred.^{12,45} Mouse lemurs show high species diversity, with at least 24 phylogenetically distinct species which have unique and disparate ranges in the remaining tropical primary and secondary forests of Madagascar.³³ Various studies have shown that one of the phylogenetically defined species, the gray mouse lemur (*Microcebus murinus*), is a valuable model of age-related neurodegeneration (for a recent review, see reference 39): a significant proportion of aged subjects exhibit progressive cerebral atrophy,³² whereas others demonstrate accumulations of amyloid

plaques and tau protein.²³ Both of these brain morphologic and biochemical abnormalities are seen in human Alzheimer disease and may be linked to cognitive decline in mouse lemurs.^{32,39} Standardized, computerized cognitive tasks comparable to the Cambridge Neuropsychological Test Automated Battery used in human diagnostics and research on psychiatric diseases, such as Alzheimer disease and schizophrenia,^{2,11} have recently been translated to the mouse lemur NHP model.^{21,37}

Worldwide, gray mouse lemurs currently are bred in 4 major captive colonies.¹² The mean lifespan in the Hannover colony, where the animal we describe here was born, is 7.7 y.⁴⁵ The genome of the gray mouse lemur has recently been sequenced, assembled, and annotated with more than 20,000 protein-coding genes (NCBI genome assembly accession GCF_000165445.2).²⁴ As a result of these various analyses, mouse lemurs represent a promising NHP model for evolutionary^{26,30,36,43,44} and biomedical research.^{12,21,25,39} Here, we report a case of spontaneous spongiform brainstem degeneration with associated abnormalities of the cerebrum and ventricles in a young female gray mouse lemur. This animal presented with prominent growth retardation, bilateral early cataracts, and conspicuous behavioral, motor, and brain structural anomalies. Immunohistochemical

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testing for an accumulation of the pathologic prion protein as a marker of transmissible spongiform encephalopathies was negative.

Materials and Methods

Housing conditions. The female animal we present was born in the mouse lemur colony of the Institute of Zoology (University of Veterinary Medicine, Hannover, Germany). Housing and colony management are in accordance with the *Guide for the Care and Use of Laboratory Animals*,¹⁹ the European Directive 2010/63/EU on the protection of animals used for scientific purposes, and the German Animal Welfare Act. The colony is approved and licensed by the official authority, the Ordnungsamt, Gewerbe- und Veterinärabteilung, Landeshauptstadt Hannover (Regulatory Agency, Department of Trade and Veterinary Concerns, State Capital Hannover; AZ 42500/1H). Husbandry practices are described in detail elsewhere.⁴⁵ In short, mouse lemurs of the colony are maintained under controlled conditions of temperature (average, 25 °C) and relative humidity (average, 50% to 60%) in cages equipped with branches, bars, and nest boxes. Throughout their lives, lemurs are maintained under a reversed dark–night cycle and an artificial photoperiodic regime (12-mo cycle) with a long-day period (14 h light, 10 h dark) for 8 mo of the year (starting in January) and a short-day period (10 h light, 14 h dark) for the remaining 4 mo (starting in September). Light is provided by using cool fluorescent lamps (360 to 780 lx) during the day and dimmed red light (4 to 19 lx) during the night. Animals are fed daily with either a mixture of fresh fruits and vegetables or a mash made of fresh bananas with milk-free rice flakes and banana milk powder (Milupa Nutricia, Bad Homburg, Germany), enriched with proteins and vitamins and supplemented 3 times each week with insects (mealworms, locusts; for details, see reference 18). Water is provided without restriction.

Behavioral and sensory examination. As part of the general husbandry routine within the breeding colony, the lemur we present was regularly weighed, and size measurements were taken by a veterinarian. During these handling sessions, the veterinarian also documented the motor behavior of the case animal. To assess sensory functioning, the animal's reaction to sounds (for example, jingling keys) and visual movements (for example, following a moving cotton ball) was checked. Further behavioral observations were made regularly by the staff veterinarian while the case animal was in its home cage. A thorough ophthalmic examination was performed once (at postnatal day [P]113) by using routine methods.^{9,10}

Anatomic analyses. After the condition of the case animal had noticeably deteriorated on P125, the decision was made to perform MRI scans of the animal's brain to make a prognosis and to decide on a potential treatment or euthanasia. Scans were conducted by using a 7-T scanner equipped with a high-performance gradient system (300 mT/m at 100 A; ramp time, 50 µs; model 70/16 Pharmascan, Bruker, Billerica, MA). The lemur was anesthetized by using 1% to 3% isoflurane in oxygen through a nose cone, and its vital signs (respiratory rate, body temperature, and heart rate by ECG) were monitored continuously by using a magnetic resonance-compatible physiologic monitoring system (model 1025, SA Instruments, Stony Brook, NY). The animal was placed in a 72-mm volume coil for signal transmission, and a quadrature surface coil was placed on its head for signal reception. T2-weighted scans were acquired by using a rapid acquisition with relaxation enhancement sequence (repetition time, 2500 ms; effective echo time, 33 ms; 8 echoes per image; 4 averages; 22 coronal and 22 axial slices;

slice thickness, 0.8 mm; field-of-view, 2.56 cm × 2.56 cm; and in-plane resolution, 100 µm × 100 µm). The animal died spontaneously during the MRI scans (after approximately 30 min). After the scans, the animal was transferred directly to the pathology department and submitted for necropsy. After macroscopic necropsy, organs were fixed in 10% neutral buffered formalin for 24 h and embedded in paraffin, and sections were stained with hematoxylin and eosin. In particular, sections of the respiratory tract (nasal mucosa, pharynx, trachea, and lungs), digestive tract (tongue, esophagus, stomach, small, and large intestine, liver and pancreas), genitourinary tract (kidneys, urinary bladder, ovaries, and uterus), endocrine system (pituitary gland, thyroid glands, and adrenal glands), integument, and bone marrow as well as both eyes were investigated. Furthermore, sections from the skeletal muscles were evaluated. Special emphasis was put on the CNS: the cerebrum, cerebellum, and brainstem were sliced in 2-mm sections and completely embedded in paraffin. Transverse sections of the cervical, thoracic, lumbar, and sacral spinal cord and longitudinal sections of the sciatic and optic nerves were prepared.

Immunohistochemical analysis. To detect depositions of pathologic prion proteins, an immunohistologic analysis of the brainstem was performed as described earlier.¹⁵ Prior to this, the reactivity of the applied prion protein detection antibody (monoclonal antibody L42¹⁶) with the prion protein of lemurs was confirmed by Western blot (data not shown). Sections of cattle brain positive and negative for bovine spongiform encephalopathy were used as controls for the immunohistochemical staining. Briefly, 3-µm sections were pretreated for 30 min in 98% to 100% formic acid, followed by blocking of endogenous peroxidase with 3% (v/v) H₂O₂ (Merck, Kenilworth, NJ) in methanol for 30 min at room temperature and incubation in 4 µg/mL proteinase K for another 15 min at 37 °C. As a primary antibody, the prion-protein-specific monoclonal antibody L42 (0.1 µg/mL) was incubated for 30 min at room temperature. Visualization was achieved by using the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, VT) and diaminobenzidine (DAB Substrate Kit). Sections were counterstained with Mayer hematoxylin, dehydrated, and mounted by using Entellan (Merck, Darmstadt, Germany). All sections were examined under light microscopy.

Genomic sequencing and analysis. DNA was extracted from liver tissue obtained from the recently dead animal by using a standard phenol–chloroform extraction method. We then performed whole-genome sequencing to investigate possible genetic causes for our findings. For this purpose, we constructed a standard paired-end PCR-free DNA sequencing library as described previously.⁴¹ Briefly, DNA was sheared, size-selected to approximately 250 to 400 base pairs, end-repaired, and ligated to molecular barcoded adaptors. This library was sequenced in a single lane (Hi-Seq X, Illumina, San Diego, CA), producing 150-basepair paired-end reads. These reads were mapped to the Mmur_2.0 genome assembly (NCBI genome assembly accession, GCA_000165445.2)²⁴ by using BWA-mem.^{27,28} Single-nucleotide polymorphisms and small insertion–deletion variants were identified by using GATK software and hard filters.⁴¹ We then used Ensembl VEP software³¹ to annotate observed variants and PolyPhen-2¹ and SIFT²² to identify variants with predicted damaging effects on protein function. The full list of candidate genes targeted for analysis included *B3GNT1*, *B3GALNT2*, *CACNA1A*, *DAG1*, *FKRP*, *FKN*, *GMPPB*, *GTDC2*, *ISPD*, *LARGE1*, *LARGE2*, *POMGNT1*, *POMGNT2*, *POMT1*, *POMT2*, *RAB3GAP1*, *RAB3GAP2*, *RAB18*, *SGK196*, *TBC1D20*, and *TMEM5*.

Case Report and Results

Age and genealogy. The female gray mouse lemur whose case is presented here was maintained with its mother until the infant died 125 d after birth during MRI brain scanning. The lemur was born and developed during the photoperiodic long-day season, as is usual for mouse lemurs.⁴⁵ Both parents are still alive at the time of this writing (February 2018). The mother was diagnosed with cataracts 8 mo after the case animal died. At that time, the mother was 4.9 y old, when age-related cataracts generally start to appear in mouse lemurs.¹⁰ Neither parent showed any conspicuous deficits in social behavior, other behavioral traits, or motor function, including behavioral assessments in open-field and novel-object tests (data not shown). However, the case animal's mother had an unusual reproductive career in the Hannover colony. Despite being paired with the father of the case animal, she never reproduced successfully during 11 estrous periods across 4 y nor when paired with another male during 3 estrous periods in 1 y. She gave birth only once (to the case animal) and showed evidence of 4 abortions divided between both males. Therefore, except for the described case, this mother did not produce living offspring, which is uncommon in this colony.

Feeding behavior. The case animal fed mainly on milk from its lactating mother. The mother lactated for an unusually long time, that is until about 3 mo after birth. However, from P35 onwards, the infant also licked from an offered milk-pap protein diet. This diet was offered daily after the infant was weaned, because it seemed to have difficulties in swallowing. In addition, it was never seen to feed on the fruits, vegetables, or insects that were offered.

Growth retardation and external appearance. The lemur had a body mass of 8.7 g at day P5, which is within the normal range for this species;⁴⁵ however, it weighed only 16 g at death (P125). In comparing the increase in body mass of the case animal with other newborn females from the birth cohort of the same year ($n = 7$), a conspicuous difference in weight began at about 9 d after birth (Figure 1 A). The typical gray mouse lemur is weaned about 35 d after birth, and the mean body weight at weaning is 35.9 g for females ($n = 20$).⁴⁵ The case female lemur weighed less than half that amount (16 g) at day P125, and it maintained the external appearance of a newborn infant—with the torso being disproportionately small in relation to the head (Figure 1 B and C)—until death, thus explaining the comparably low body weight. In addition, 2 wk before death, the dorsal fur of the lemur became thin.

Sensory impairments. The pupillary reflex of the case animal was normal (that is, less than 2 s). Incipient cataract was detected in both eyes in the anterior lens chamber, and the anterior lens epithelium of the left eye showed abnormal pigmentation. Details of the retina in the left eye were difficult to investigate due to cataract and pigmentation. The typical yellow–orange color of the tapetum lucidum in mouse lemurs could not be detected. Instead, the retina showed a more reddish color without reflections, as is typical for animals that lack a tapetum lucidum. The intraocular pressure was within the normal physiologic range,⁹ but the animal did not show optokinetic nystagmus toward objects, such as a finger or a white cotton ball, moving in front of its face, thus suggesting some form of vision impairment. Until the case we describe here, bilateral incipient cataracts had not been observed in mouse lemurs younger than 1 y in either our colony or another large European colony.⁹ The case animal was noted to exhibit pinnal reflexes toward acoustic signals and answered to calls of its mother, thus suggesting that it was able to hear normally.

Movement disorders. In comparison to same-aged mouse lemurs, the case animal exhibited a similar ability to walk and climb but was unable to jump. At the age of 4 mo, shortly before the MRI scan, it developed severe motor dysfunction, including weakness of hindlimbs and a pattern of suddenly falling from branches or bars to the ground. In addition, it displayed severe ataxia and tremors, lasting as long as 6 min. As mentioned earlier, the case animal appeared to have difficulties in feeding and swallowing, and these abnormalities prompted the veterinary staff's decision to perform MRI of the brain for further diagnosis and for identifying potential treatment.

MRI findings regarding brain morphology. The T2- and T2*-weighted imaging sequences revealed signal hyperintensities, consistent with an increased water signal affecting the ventral hippocampus, right lateral ventricle, dorsal third ventricle, and aqueduct (see reference 4 for a brain atlas of *M. murinus*). In addition, hypointense signal was apparent in the right hemisphere, spanning from the area of the caudate nucleus and corpus callosum to the dorsal thalamic nuclei. The hypointense rim on T2-weighted images (Figure 2) often relates to the magnetic susceptibility variations of paramagnetic deoxygenated hemoglobin.⁴² However, routine histology did not demonstrate any hemorrhage or other described causes for hypointense, intracranial T2 lesions (for an overview, see reference 46).

Necropsy findings. The case animal was investigated macroscopically after death. It was in poor nutritional condition. Macroscopic abnormalities were present in the reproduction system and brain. In the reproductive system, the left uterine horn was present as only a thin, fibrous cord, and the left ovary was absent. The right uterine horn and ovary showed typical development. The brain showed mild unilateral dilation of the right lateral ventricle, with mild compression of the overlying cerebral cortical parenchyma (compare with the MRI findings described earlier). The other organs were macroscopically unremarkable.

Histologic and immunohistochemical findings. Bilaterally within the brainstem, beginning from the colliculus and extending to the medulla oblongata, large, vacuolated areas were present (Figures 3 and 4). The vacuoles were mainly located in the white matter and the funiculi, but single neurons of the brainstem nuclei also contained large, sharply demarcated, optically empty intracytoplasmic vacuoles, with margination and compression of the nucleus (Figures 3 and 4). Staining of the brain tissue by using Congo red and Luxol fast blue–Cresyl echt violet staining, periodic acid Schiff reaction, and immunohistochemistry for amyloid precursor protein was performed but revealed no additional results. The other organs did not show significant microscopic lesions. Furthermore, immunohistochemical analysis of brainstem sections of the case lemur did not reveal any deposition of pathologic prion protein.

Genetic findings. On the basis of the comparison of genomic sequence data from the presented mouse lemur and the reference genomic sequence for this species (Mmur2.0, GenBank accession no., GCA_000165445.2),²⁴ we detected several potentially functional protein-coding variants. To narrow the search for potential causes, we searched the case genomic sequence for mutations that are potentially damaging (that is, de novo stop codons, splice-site mutations, nonsynonymous variants, or small insertion–deletion mutations) and that were present in genes known or suspected to cause comparable clinical phenotypes in humans. The list of genes screened is presented in the Methods section.

We found one nonsynonymous (that is, amino-acid-changing) variant in the gene *DAG1* and another nonsynonymous

Figure 1. Growth retardation and external appearance. (A and B) Growth curves of the case female lemur (solid red line) as compared with the other females born in the colony in the same year (solid black lines). (A) Body weight. (B) Torso length (from neck to tail base). (C) Photographs of the case animal (top) and a normally developed female infant (bottom) at postnatal day 100.

Figure 2. Structural MRI at postnatal day 125. (A) Representative T2-weighted scans from the axial sequence (from rostral to caudal); distance between example slices, 1.6 mm. (B) Representative T2-weighted scans from the coronal sequence (from dorsal to ventral); distance between example slices, 1.6 mm. Conspicuous signal hyperintensities spanning large parts of the brain can be seen in the region of the right lateral ventricle (red arrowheads); a prominent hypointensity can be found in the tissue adjacent to the right lateral ventricle (cyan arrowheads). (C) MRI-based, 3D reconstruction of the brain from a frontal view.

variant in *POMGNT2*. Both of the mutations in *DAG1*³⁵ and *POMGNT2*,²⁹ which is also called *GTDC2*, have been associated with Walker–Warburg syndrome in humans. However the widely used mutation analysis programs SIFT and Polyphen-2 both scored these 2 variants as relatively benign and unlikely to have damaging effects on protein function. In addition, we found a nonsynonymous variant in the gene *LARGE2*, a gene that is also called *GYLTL1B*. The variant in *LARGE2* was scored as probably damaging by Polyphen-2 and deleterious by SIFT and was not present in any of the 10 additional *M. murinus* animals for which we have whole-genome sequences. Mutations in the highly similar gene *LARGE1* (previously called *LARGE*) are known to cause Walker–Warburg syndrome.³⁸ *LARGE2* is recognized as having very similar function to *LARGE1* and has been suggested as a possible candidate for Walker–Warburg syndrome and other muscular dystrophies.⁵ Finally, we found that the case animal also carried 2 damaging mutations in *CACNA1A*, which is not known to cause Walker–Warburg syndrome

or closely related disorders but has been associated with ataxia and epilepsy in humans.^{6,34} We found a nonsynonymous mutation and a potential frameshift deletion in this gene.

Discussion

The mouse lemur we present showed a complex clinical phenotype with spontaneous spongiform brainstem degeneration and several behavioral and motor signs, including impaired motor skills, severe ataxia, and tremors, as well as missing optokinetic nystagmus toward moving objects. The oculomotor disturbances, as well as the other motor signs, likely were linked to the most prominent neuropathologic finding, the vacuolar and spongiform changes in the brainstem. Whether the second neuropathologic finding, dilation of the right lateral ventricle, is an associated lesion or independent of the brainstem vacuolization remains undetermined.

Apart from transmissible forms or familial diseases, spongiform changes unrelated to prion disorders have been described

Figure 3. Brain mapping indicating the morphologically affected areas in the CNS. Anatomically differentiable brain structures, such as nuclei and specific fasciculi, are highlighted in gray. (A) Transverse section at the level of the colliculus. Red stars, vacuoles in the white matter; cyan stars, vacuoles in neurons at ventral aspects of the inferior colliculus. (B) Transverse section at the level of the nucleus cuneatus or ambiguus. Red stars, vacuoles in the white matter; cyan stars, vacuoles in neurons, mainly affecting the nucleus tractus spinalis nervi trigemini and the nucleus reticularis ventralis and lateralis. Regions were identified as previously described.⁴

Figure 4. Histopathologic findings in the CNS. (A) Brainstem. There is severe, bilateral vacuolation of the white matter (arrows) and funiculi and evidence of vacuoles within the cytoplasm of single neurons (arrowheads). On the right side, the aqueductus mesencephali is visible (star). (B) Colliculus. Multifocally, at the section level of the colliculus, there is severe vacuolation of the white matter tracts (arrows) as well as evidence of large vacuoles within single neuronal perikarya (arrowheads). Affected neurons show severe marginalization of the nucleus. Hematoxylin and eosin staining (A and B).

to occur due to a variety of metabolic defects¹⁷ as well as in geriatric animals.^{14,20} A case of spontaneous transmissible spongiform encephalopathy in a 4-y-old rhesus monkey potentially originated from cross-species transmission through contaminated feed,³ similar to the mechanism suggested for bovine spongiform encephalopathy; spontaneous spongiform degeneration in the brainstem of a NHP has not otherwise been described to date. In our mouse lemur, immunohistochemistry did not reveal any deposition of pathologic prion protein, and foodborne transmission of prions is highly unlikely, given our lemur's young age and the fact that no other animal in the colony had any clinical signs. We, therefore, exclude a prion infection as a possible cause for the histopathologic changes. The

features of the spontaneous spongiform lesions in the mouse lemur we present show important differences from other forms of spongiform encephalopathies, especially transmissible forms, and must be considered indicative of a unique and so far pathogenetically undetermined disease.

The mouse lemur we present further showed several developmental abnormalities, including severe growth retardation, maldevelopment of the left uterine horn and ovary, abnormal pigmentation in the lens of the left eye, and incipient bilateral cataracts. These developmental disturbances may be metabolic (linked to early malnutrition) or genetic in origin, or both. Conspicuous genetic features of the case were potentially pathologic mutations in *DAG1*, *POMGNT2*, *LARGE2*, and *CACNA1A*.

Mutations in 2 of these proteins, DAG1 and POMGNT2, are directly linked to Walker–Warburg syndrome,^{29,35} a severe, progressive muscular dystrophy.⁴⁰ LARGE2 has no such direct connection to Walker–Warburg syndrome, but its close homolog LARGE1 does.¹³ The pattern of tissue-specific expression in humans differs for LARGE1 and LARGE2, but their distribution has not been analyzed extensively in mouse lemurs, in which LARGE2 may have specific functions or expression patterns that are more characteristic of LARGE1 in humans. This issue requires further study but is beyond the scope of this report. The fourth mutation we found in *CACNA1A* is not known to be involved in dystroglycanopathies, and we consider it to be less likely as a candidate for a causative mutation.

Walker–Warburg syndrome⁴⁰ is a progressive muscular dystrophy that usually leads to death within the first years of life.¹³ Additional signs usually comprise severe brain and eye anomalies (including congenital cataracts) as well as highly disturbed or absent motor development. Furthermore, some patients with Walker–Warburg syndrome have been reported to exhibit feeding difficulties and low visual attention.¹³ Our case animal, therefore, shared several genotypic and phenotypic similarities with human patients with Walker–Warburg syndrome. Our mouse lemur's falls from branches, inability to jump, and refusal of solid food items might be indications of incipient skeletal muscle weakness, but malformation of skeletal muscles at a macroscopically detectable level, as a prominent sign of Walker–Warburg syndrome and related muscular dystrophies, was absent at the time of death. Moreover, routine histology failed to reveal any morphologic indication for any form of muscular degeneration or atrophy. Given that mouse lemurs are lissencephalic NHP,⁸ the presence or absence of a second prominent feature of Walker–Warburg syndrome—cobblestone lissencephaly—could not be assessed in the case animal. The most severe subtype of this cortical malformation in humans has been linked to mutations in *POMT1*, *POMT2*, and *FKRP*,⁷ none of which showed any potentially pathologic mutations in the case we describe here. With regard to LARGE family members, only LARGE1 has been linked to cobblestone lissencephaly in humans,⁷ and even then to a less severe form (subtype B) in which ventricular dilation (especially of ventricle 4) and brainstem malformations are common, but agyria is not necessarily observed.⁷ In conclusion, we suggest that the mutations in *LARGE2*, *DAG1*, and *POMGNT2* of the case animal were contributing factors—individually or in concert—in the observed developmental pathologies. Although the predicted effects (according to SIFT and Polyphen-2 analyses) of the mutations in *DAG1* and *POMGNT2* were not as severe as that for the *LARGE2* variant, mutations that may be relatively benign in humans might be more damaging to protein function in mouse lemurs. However, it is not possible at this time to assign this mouse lemur case to a specific disorder homologous to human Walker–Warburg syndrome or a related, less severe syndrome.

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