A mouse model for eukaryotic translation initiation factor 2B-leucodystrophy reveals abnormal development of brain white matter

Michal Geva,1,*,† Yuval Cabilly,1,*, Yaniv Assaf,2 Nina Mindroul,1 Liraz Marom,1 Gali Raini,1 Dalia Pinchasi1 and Orna Elroy-Stein1

1 Department of Cell Research and Immunology, George S. Wise Faculty of Life Science, Tel Aviv University, Tel Aviv, Israel
2 Department of Neurobiology George S. Wise Faculty of Life Science, Tel Aviv University, Tel Aviv, Israel
*These authors contributed equally to this work.
†Present address: Institute for Neurodegenerative Diseases, University of California at San Francisco, CA, USA

Correspondence to: Orna Elroy-Stein,
Department of Cell Research and Immunology,
George S. Wise Faculty of Life Science,
Tel Aviv University, Tel Aviv, Israel
E-mail: ornaes@tauex.tau.ac.il

Eukaryotic translation initiation factor 2B is a major housekeeping complex that governs the rate of global protein synthesis under normal and stress conditions. Mutations in any of its five subunits lead to leucoencephalopathy with vanishing white matter, an inherited chronic-progressive fatal brain disease with unknown aetiology, which is among the most prevalent childhood white matter disorders. We generated the first animal model for the disease by introducing a point mutation into the mouse Eif2b5 gene locus, leading to R132H replacement corresponding to the clinically significant human R136H mutation in the catalytic subunit. In contrast to human patients, mice homozygous for the mutant Eif2b5 allele (Eif2b5R132H/R132H mice) enable multiple analyses under a defined genetic background during the pre-symptomatic stages and during recovery from a defined brain insult. Time-course magnetic resonance imaging revealed for the first time the delayed development of the brain white matter due to the mutation. Electron microscopy demonstrated a higher proportion of small-calibre nerve fibres. Immunohistochemistry detected an abnormal abundance of oligodendrocytes and astrocytes in the brain of younger animals, as well as an abnormal level of major myelin proteins. Most importantly, mutant mice failed to recover from cuprizone-induced demyelination, reflecting an increased sensitivity to brain insults. The anomalous development of white matter in Eif2b5R132H/R132H mice underscores the importance of tight translational control to normal myelin formation and maintenance.

Keywords: CACH; VWM; eIF2B; leucodystrophy; mouse model
Abbreviations: CACH = childhood ataxia with central nervous system hypomyelination; eIF2B = eukaryotic translation initiation factor 2B; GFAP = glial fibrillary acidic protein; NG2 = chondroitin sulphate proteoglycan; PLP = proteolipid protein

Introduction

Eukaryotic translation initiation factor 2B (eIF2B), a major component of the translational machinery, is an evolutionary-conserved housekeeping complex that governs the rate of global protein synthesis under normal and stress conditions. It serves as the guanine nucleotide exchange factor of eukaryotic translation initiation factor 2 (eIF2). In its guanosine triphosphate-bound
form, eIF2 loads the initiator Met-tRNAi onto the small ribosomal subunit, followed by release of eIF2-guanosine diphosphate at each round of translation initiation (Sonenberg and Dever, 2003). Under various stress conditions, the α-subunit of eIF2 is phosphorylated by one of four kinases, converting eIF2 from a substrate into a competitive inhibitor of eIF2B and leading to inhibition of global protein synthesis (Dever, 2002). Due to this essential role in messenger RNA (mRNA) translation under normal and stress conditions across all cell types, it is surprising that a wide range of mutations in each of the five subunits of Eif2B specifically lead to a neurodegenerative disease that primarily affects the white matter of the CNS (Leegwater et al., 2001). This disorder, previously described as diffuse white matter disease or childhood ataxia with CNS hypomyelination (CACH) or vanishing white matter, was later termed eIF2B-related leucodystrophy or childhood ataxia with CNS hypomyelination (CACH) or vanishing white matter, was later termed eIF2B-related leucodystrophy (OMIM #306896) (Leegwater et al., 2001; Hanefeld et al., 1993; Schiffmann et al., 1994). It is recognized as one of the most prevalent inherited childhood white matter disorders and also affects individuals of all ages with a wide phenotypic variation related to type of mutation, genetic background and environmental stressors, jointly affecting disease onset and symptom severity (van der Knaap et al., 1998; Fogli and Boespflug-Tanguy, 2006). The classical form of the disease is associated with a progressive loss of myelin in the CNS, leading to neurological motor and cognitive deficits. Episodes of severe clinical deterioration are often observed upon exposure to various stressors, such as head trauma, febrile illness and acute fright. Diagnostic MRI of the brain usually shows abnormal T1- and T2-weighted signals in the white matter indicative of tissue degeneration and infiltration of CSF (Schiffmann and Elroy-Stein, 2006; van der Knaap et al., 2006). It is currently unclear how eIF2B mutations cause a white matter disorder. Diffusion imaging data for very early asymptomatic stages of the disease are not available and little is known about the developmental component of this disorder. Here, we report the generation of a mouse strain homozygous for a mutation in the catalytic subunit of Eif2b (Eif2b5R132H/R132H mice), which constitutes the first animal model for EIF2B-related leucodystrophy. Time-course measurements of diffusion and T2 changes using these mice provide compelling evidence that hypomorphic Eif2b5 alleles lead to delayed and abnormal development and maintenance of CNS white matter.

Materials and methods

Construction of Eif2b5(R132H) targeting vector

A 2.4-kb SpeI–SnaB1 fragment, extending from the promoter region to the SnaB1 site within intron 2 of the mouse Eif2b5 gene (accession NM_172265), and a 4.8-kb SnaB1–Xba1 fragment, extending from the same site in intron 2 to intron 9 of the same gene, were isolated from a λFIX II mouse genomic clone originated from a 129/SvEv genomic library (Stratagene, La Jolla, CA, USA). The two fragments were used as the 5′ and 3′ arms, respectively, to construct the gene-targeting vector. Prior to the insertion of the 3′ arm to the targeting vector, it was first subcloned into SnaB1–Xba1 sites of pcDNA3.1 (Invitrogen) to generate the G2723A and T2756C mutations within exon 3. The G2723A mutation was designed to replace the arginine-encoding CGT codon into histidine-encoding CAT codon. The silent T2756C mutation (as both GAT and GAC codons encode asparagine at position 135) was designed to generate a unique SaI site within the mutated allele. To generate the mutations, three polymerase chain reactions were performed as follows: primers 5′-GGCTTCCGTGTATTTGATC-3′ and 5′-CTTGCGTCGACGTC ATGGAG-3′ were used to generate a 968-bp fragment; primers 5′-CTCCATACGTGCACGCCAG-3′ and 5′-GTGAGAAAACAGA GCACCCG-3′ were used to generate a 695-bp fragment; finally, both polymerase chain reaction products were used for the third reaction using primers 5′-GGCTTCGTCGATTTTGATC-3′ and 5′-GTGAGAAAACAGA AACAGACCCG-3′ to obtain the desired mutated SnaB1–Kpn1 1557-bp fragment, which replaced the corresponding SnaB1–Kpn1 fragment lacking the mutations. The generated pcDNA3.1 plasmid containing the mutated 3′ arm was termed ‘pc-III-MUT’. The two arms were cloned at both sides of the Neo cassette in the pOSDupDel.Neo targeting vector (Open biosystems). The 5′ arm 2.4 kb SpeI–SnaB1 fragment was inserted between NheI–Pml1 sites, and the mutated 3′ arm 4.8 kb SnaB1–Xba1 fragment was inserted between HpaI–Xba1 sites, yielding the targeting vector p2BS.KI.R132H.

Generation and maintenance of Eif2b5(R132H) mutant mice

Gene-targeted 129/SvEv embryonic stem cells were produced by electroporating the Pme1 linearized p2BS.KI.R132H targeting vector (inGenious Targeting Laboratory, Inc.). Sph1 digested genomic DNA from G418 and Gancyclovir resistant clones was screened by Southern blot analysis using 32P-labelled 5′ and 3′ external probes that flank the targeted locus and detect a 13-kb wild-type fragment and a 6.1-kb mutated fragment or a 13-kb wild-type fragment and a 6.8-kb mutated fragment, respectively (Fig. 1). The 5′ probe was amplified from genomic DNA using primers 5′-GTGACCTACA TATGTTGAGGC-3′ and 5′-GGAAAATCTGCTAATGGAGATC-3′, whereas the 3′ probe was amplified using primers 5′-TTTGACCTT CAGTTTACATCTGAACG-3′ and 5′-GGGTTAATATATCTCTTTCTGCT CAAAC-3′. Two correctly targeted heterozygote embryonic stem cell clones were injected into C57BL/6 blastocystcs (inGenious Targeting Laboratory, Inc.), and chimeric males that transmitted the mutant allele through their germline were obtained from both lines. Animals were genotyped by polymerase chain reaction using primer int2 (5′-CTCTTTGAAACCGGAGGC-3′, complementary to the intrinsic sequence upstream of exon 3) and primer ex3 (5′-GA AGGTCTTCTACACCTCC-3′, complementary to the 3′ end of exon 3) followed by Sal1 digestion. The wild-type fragment is refractory to Sal1 digestion, whereas the mutated fragment yields 880-bp and 220-bp fragments. F1 heterozygotes were crossed to produce F2 eIF2B5+/− (wild-type) and eIF2B5−/− (mutant) that were used for mating to produce the offspring used in this study. In all studies comparing wild-type and mutant mice, except for the motor function testing, we used male siblings derived from the above mating. The eIF2B5 mutation is maintained in two different genetic backgrounds: inbred C57BL and backcross to the outbred Swiss Webster strain. For motor function testing, we used wild-type and mutant siblings of heterozygote mice that were backcrossed to the C57BL strain for six generations. All experimental procedures involving mice were approved by the Tel Aviv University Animal Care Committee. Mice
were housed in an animal facility with a 14/10 h light/dark cycle in groups of four to seven animals in each filtered-top cage supplemented with autoclaved wood chips in laminar flow hoods. Animals were fed autoclavable rodent pellet (Koffolk 19–510, Koffolk Ltd, Petach Tikva, Israel) and sterile water ad libitum throughout the experiments. To induce demyelination, 6-week-old male mice were fed a diet of milled mouse chow supplemented with 0.2% cuprizone (Sigma) for 4 weeks (Matsushima and Morell, 2001). Remyelination was initiated by returning the mice to a normal diet for an additional 4 weeks.

Refer to the online supplementary material for methods of eIF2B activity assay, polysomes profile analysis, electron microscopy, motor function assays, fat mass analysis, histochemistry and immunostaining, myelin purification, immunodetection and MRI analysis.

**Results**

**Targeting of the G2723A (R132H) mutation into the Eif2b5 mouse gene locus and evaluation of the mutated eIF2B enzymatic activity**

To create an animal model for eif2b5-related leucodystrophy, we generated a mutant mouse strain by introducing a specific point mutation into the gene locus encoding subunit 5 of Eif2b. The change of guanine to adenine at position 2723 leads to the replacement of a conserved arginine by histidine at position 132 of
the protein. The murine R132H mutation corresponds to the human R136H mutation, which is associated with a classical form of CACH/vanishing white matter disease when present at a homozygous state (Kantor et al., 2005). A targeting vector containing portions of murine Eif2b5 gene locus was designed to introduce a neomycin resistance cassette into intron 2 while replacing exon 3 with a genetically engineered exon 3 that harbours the G2723A mutation and a silent mutation generating a novel and unique Sal1 restriction site to facilitate genotyping (Fig. 1A). Genotyping was performed by Southern blot analysis using probes from both 5’ and 3’ flanking regions and by polymerase chain reaction (Fig. 1B–D). Breeding of heterozygous mice produced offspring consistent with the expected Mendelian distribution, without any alteration of the male-to-female ratio (data not shown). Mice homozygous for the R132H mutation (Eif2b5R132H/R132H, hereinafter referred to as mutant mice) were viable and fertile.

Western blot analysis did not detect any significant decrease in Eif2b5 protein levels in either the cerebrum or cerebellum of the mutant mice (Fig. 1E). However, eIF2B enzymatic activity in cerebrum of mutant compared with wild-type mice, detected by exchange of eIF2-GDP to eIF2-GTP, was reduced by 23% (Fig. 1F). This is the first demonstration that a mutation associated with a human classical form of CACH/vanishing white matter reduces Eif2b5 enzymatic in the brain. However, the mutation was not found to have any significant effects on global protein synthesis as assayed by polysomal profile analysis of total brain RNA (Supplementary Fig. 1).

**Impaired motor functions and altered growth rate**

Under normal conditions, the mutant mice exhibited a normal life span and did not develop severe clinical symptoms. This is in contrast to the human patient homozygous for the corresponding mutation (R136H), who was diagnosed at the age of 3 years, developed progressive neurological deterioration and died within 5 years of diagnosis. However, although the mice remained active in their cages throughout ageing without developing paralysis, they did exhibit impaired motor functions reflected in a rotarod test. Motor deficit in the rotarod test was apparent as early as at the age of 6 weeks, without further improvement in rotarod scores as the mice grew older, in contrast to wild-type controls (Fig. 2A). In addition, to test spontaneous activity, we followed the performance in an open-field assay, commonly regarded as a fundamental index of general behaviour, also predictive of locomotor scores (Walsh and Cummins, 1976). Each wild-type or mutant mouse was introduced into a large square plain arena for 30 min, during which mutant mice travelled a lesser total distance compared with wild-type mice. The individual scores of the open-field assay are shown in Fig. 2B, clearly demonstrating that all mutant mice aggregate at the relatively low-travelled distances, whereas the wild-type mice aggregate at the high-travelled distance. The mean distances for both groups differ by more than 25% of the data range ($P=0.0006$), indicative of their impaired motor function.

In contrast to patients with CACH/vanishing white matter, whose growth rate and average body weight is considered normal, time-course measurements throughout the life of the mice revealed that the average body weight of mutant mice was 10–15% and 24% lower at the age of 6–40 and 90 weeks, respectively, compared with wild-type mice (Fig. 3A). Body mass revealed that the average body weight of mutant mice whose growth rate and average body weight is considered normal, time-course measurements throughout the life of the mice revealed that the average body weight of mutant mice was 10–15% and 24% lower at the age of 6–40 and 90 weeks, respectively, compared with wild-type mice (Fig. 3A). Body mass analysis by dual X-ray absorptiometry at the age of 90 weeks revealed a significantly lower body fat percentage in mutant mice (20.2% versus 11.5% fat in wild-type and mutant mice, respectively) (Fig. 3B).

**Abnormal time-course MRI**

MRI is diagnostic for CACH/vanishing white matter disease. Therefore, MRI was used to define an in vivo whole-brain quantitative phenotype at different ages. Each group of mutant and wild-type male mice was subjected to multiple brain scans at several time points between the ages of 3 and 90 weeks. Analysis of
the quantitative MRI indices was performed using voxel-based analysis, which allows a whole brain comparison. This analysis demonstrated that affected regions were the white matter systems (corpus callosum and motor/sensory pathways) and the hippocampus; therefore, regional analysis was focused in those regions. Under normal growth conditions, abnormal white matter development was detected in mutant compared with wild-type mice. While the conventional quantitative T2 values in the internal capsule of both groups were similar, the T2 values in lower motor regions of the mutant mice, i.e. cerebral peduncles and brain stem, increased with age and ultimately reached a level higher than that of the wild-type group (data not shown). To characterize the integrity of the white matter and monitor the progress of mild changes in tissue composition better, we employed diffusion tensor imaging, which allows quantification of water diffusion. The indices extracted from diffusion tensor imaging serve as microstructural indices that can detect regional differences in white matter (Ashburner and Friston, 2000). The common-most diffusion tensor imaging parameters include fractional anisotropy (a marker of tissue organization), apparent diffusion coefficient (a marker of cellular integrity) and radial and axial diffusivity (markers of white matter fibre density and organization, respectively) (Basser and Jones, 2002; Assaf, 2008). Normal developmental process is characterized by an increase in fractional anisotropy and decrease in apparent diffusion coefficient with age. Typical degenerative process is characterized by a decrease in fractional anisotropy and increase in apparent diffusion coefficient. To monitor developmental abnormalities, each group of mutant and wild-type male mice was subjected to multiple brain diffusion tensor imaging analyses at several time points between the ages of 3 and 90 weeks. Statistical parametric maps representing the fractional anisotropy differences between the groups (wild-type > mutant) at the ages of 3, 12 and 90 weeks demonstrated a significant difference in the internal capsule and other regions, including the external capsule, septum and caudate/putamen at 3 weeks of age; this difference became less significant as the mice grew older (Fig. 4). Analysis of the statistical parametric maps for all age groups shows that whereas the fractional anisotropy of wild-type internal capsule did not change throughout the experiment, the fractional anisotropy of mutant internal capsule was significantly lower at 3 weeks of age and reached wild-type level only at 90 weeks of age (Fig. 5A). A similar trend was observed for the external capsule, septum and caudate/putamen (data not shown). Lack of growth-dependent fractional anisotropy changes in the wild-type internal capsule beyond the age of 3 weeks seems to indicate that the development of this region is normally completed by this age. In contrast, the low fractional anisotropy values of mutant internal capsule indicate delayed development in association with Eif2b5 mutation. Analysis of the statistical parametric maps for the corpus callosum region in all age groups shows a similar trend, albeit with lower statistical significance (Fig. 5B). The apparent diffusion coefficient value of the internal capsule in both groups was similar at the age of 3 weeks, which might indicate similar tissue densities. However, the steady age-dependent increase in apparent diffusion coefficient values observed in the mutant but not wild-type internal capsule (Fig. 5C) indicates a process of decreasing tissue density in a region critical for motor functions. Taken together, these data demonstrate that Eif2b5 mutation is associated with delayed development of the internal capsule region based on fractional anisotropy values, which were normalized at older age. However,
Figure 4 Voxel-based analyses of regionally specific time course changes in fractional anisotropy. Seven males in each wild-type (wt) or mutant (mut) group were subjected to live MRI at the indicated ages. Statistical parametric maps of the comparison FA(wt) > FA(mut) at the ages of 3, 12 and 90 weeks are shown for two Bregma positions as indicated. Regions that passed the voxel-based analysis statistical threshold (taken as P < 0.01, uncorrected for multiple comparisons) are shown. The significant changes observed at the age of 3 weeks reduced as the mice grew older. FA = fractional anisotropy.

Figure 5 Region of interest analysis of age-dependent changes for fractional anisotropy and apparent diffusion coefficient. Following detection of regions that passed the statistical threshold (Fig. 4), post hoc analysis of the statistical parametric fractional anisotropy (FA) and apparent diffusion coefficient maps for all age groups was performed on selected voxels of interest. Statistics were computed using Student’s t-test (two-tailed distribution with unequal variance; bars represent the averages ± SD). White circles = wild-type; black squares = mutant. (A) Age-dependent fractional anisotropy of the internal capsule region, showing the low fractional anisotropy values of the mutant (Mut) group at young ages that reach wild-type (wt) values as the mice grew older. (B) Age-dependent fractional anisotropy of the corpus callosum region. (C) Age-dependent apparent diffusion coefficient (ADC) of the internal capsule region showing similar values for wild-type and mutant at age of 3 weeks and their dissociation as the mice grew older. Similar trend is observed in the hippocampus (D).
the normalization of fractional anisotropy values does not necessarily indicate normalization of the slowly developing fibre system, because the unexpected increase in apparent diffusion coefficient at an older age suggests a degenerative pattern. Similarly, the time-course changes of hippocampal apparent diffusion coefficient revealed a decrease in the wild-type group compared with an increase in the mutant group, indicating a gradual increase in density of wild-type and decrease in density of mutant hippocampal tissues (Fig. 5D). Opposite apparent diffusion coefficient trends for the wild-type and mutant groups were also observed for the caudate/putamen and midbrain (data not shown).

**Higher proportion of small-calibre axons in young mice and late-onset myelin degeneration**

Transmission electron microscopy analysis of two different areas of the internal capsule from each lobe of three 3-week-old wild-type and mutant mice did not show any major inter-group structural differences in myelination between the groups (data not shown) but did show increased proportion of thin axons in the mutant group (Fig. 6A). The radial diffusivity index (obtained by diffusion tensor imaging, MRI), which represents the diffusion of water perpendicular to myelin fibres, was significantly increased in the internal capsule region of mutant mice, indicating an abnormal myelination pattern (Fig. 6B). It is generally accepted that radial diffusivity correlates with myelin content (Song et al., 2005; Barazany et al., 2009). Analysis of 3200 axons from each group demonstrated a significant visible difference in axonal thickness, as confirmed by a 26% reduction in average total axon cross-section area in mutant compared with wild-type mice (Fig. 6C). Assessment of axon cross-section area distribution revealed a higher proportion of thin axons (0.15 μm² or less) in the internal capsule of 3-week-old mutants (53.6%) compared with wild-type mice (40.3%) (Fig. 6D). The axon/fibre diameter (G-ratio) for both groups was similar (wild-type: 0.747 ± 0.006; mutant: 0.73 ± 0.007; n = 100 for each group, P = 0.053). Therefore, the higher proportion of small-calibre axons implies that the overall amount of myelin in 3-week-old mutant mice is lower. Transmission electron microscopy examination of the cerebral peduncle, a significantly affected region, based on MRI analysis of older mice, demonstrated increased proportion of demyelinated fibres in 65-week-old mutants compared with wild-type mice. Moreover, the mutants contain a higher proportion of axons ensheathed by thinner myelin, with circumferential splits and redundant loops, indicative of a neurodegenerative process (Fig. 6E).

**Abnormal level of major myelin proteins**

Myelin was then probed from a developmental point of view by immunostaining for myelin basic protein and proteolipid protein (PLP)-DM20, two major CNS myelin proteins that serve in compaction of myelin sheaths. For this purpose, brain sections from 3-week- and 4-month-old male mice were immunostained. Figure 7A demonstrates a significant increase in myelin basic protein and a decrease in PLP/DM20 content in the hippocampus of young mutant mice. Myelin basic protein levels were normalized at an older age, while PLP/DM20 levels remained low. Western blot analysis established that myelin basic protein levels were higher, whereas PLP/DM20 levels were lower in 3-week-old mutant mice compared with the wild-type mice (Fig. 7B and C).

**Abnormal density of oligodendrocytes and astrocytes**

It was previously reported that brains extracted post-mortem from patients with CACH/vanishing white matter show a higher abundance of oligodendrocytes (van der Knaap et al., 1998; Rodriguez et al., 1999; Francalanci et al., 2001; Van Haren et al., 2004) and lower abundance of astrocytes (Francalanci et al., 2001; Dietrich et al., 2005). To analyse the abundance of these glial cell types from a developmental point of view, brain slices of 3-week and 4-month-old wild-type and mutant male mice were used for immunohistochemistry using antibodies for the chondroitin sulphate proteoglycan (NG2; a marker of immature oligodendrocytes) and the intermediate filament glial fibrillary acidic protein (GFAP; a marker of astrocytes). Mean integral optical density and total stained area were used as the parameters of choice for image analysis. For NG2, both parameters were significantly higher in young mutant mice compared with young wild-type mice (Fig. 8A). We noticed that within the normal integral optical density distribution, values that are 2 standard deviations (SD) above the mean represent cell bodies, while values between 0.5 and 1.5 SDs above the mean typically represent cell processes. While the integral optical density of the cell bodies revealed no significant difference between young mutant mice and young wild-type mice (P = 0.09), the area parameter was 3.4-fold higher for mutant compared with wild-type controls (P = 0.01). Since the actual body size of NG2-positive cells in both cases was similar (Fig. 8B), we concluded that 3-week-old mutant mice contain 3.4-fold more NG2-positive cells compared with wild-type controls. Interestingly, the mutant NG2-positive cells contained more processes compared with the wild-type, as quantified as a 1.4-fold increase in both integral optical density and area parameters (Fig. 8A). As the mutated mice grew older, the developmental anomaly became less and less significant. Four-month-old mutant mice contained slightly less NG2-positive cells compared with wild-type mice, with no significant change in their NG2-positive processes (Fig. 8A). It should be noted that foamy oligodendroglial cells previously found in severe forms of CACH/vanishing white matter (Wong et al., 2000; Fogli et al., 2002) were not observed in the mutant mice. Unlike oligodendrocytes, a significant decrease in the abundance of GFAP-positive cells was observed in 3-week-old mutant compared with wild-type mice (Fig. 9). Similarly, this developmental anomaly was also normalized as the mice grew older; 4-month-old mutant mice had a similar abundance of astrocytes compared with the wild-type mice, yet their GFAP level was slightly higher (Fig. 9B).
Impaired recovery from cuprizone-induced demyelination

Based on the observation that the clinical symptoms of EIF2B-leucodystrophy worsen upon exposure to various stressors (Schiffmann and Elroy-Stein, 2006; Damon-Perriere et al., 2008), it was of particular interest to assess the capacity of the mutant mice to recover from a brain insult that specifically leads to myelin damage. For this purpose, we applied the well-characterized cuprizone model for experimental demyelination and remyelination of the mouse brain. Administration of cuprizone leads to myelin vacuolation and loss that is reversible if the exposure to cuprizone is stopped on time (Matsushima and Morell, 2001). Time-course MRI scans were used to detect the progression of myelin degeneration and regeneration. The mice were fed a diet supplemented with 0.2% cuprizone for 4 weeks starting at the age of 6 weeks, resulting in a subsequent increase in T2-values in the posterior part of the corpus callosum of both groups that is indicative of a demyelination process. Upon withdrawal of cuprizone, the T2-value of the wild-type group gradually returned to baseline within 4 weeks, indicating a normal remyelination process. In contrast to the wild-type group, the T2-value of the mutant mice continued...
to rise even in the absence of cuprizone, followed by a delayed decline not reaching complete recovery, indicating delayed and abnormal remyelination (Fig. 10A). Additional diffusion tensor imaging analyses confirmed the meagre remyelination capability of the mutant mice. During the cuprizone-supplemented diet, we observed reduced fractional anisotropy and axial diffusivity and increased radial diffusivity in the corpus callosum (Fig. 10B) and internal capsule (data not shown) of wild-type and mutant mice. During recovery, these indices were normalized in wild-type mice, inline with previous observations (Song et al., 2005) and consistent with a remyelination process. However, whereas the wild-type mice completely recovered within 4 weeks, improvement was only partial in the mutant internal capsule (data not shown) and undetectable in the mutant corpus callosum (Fig. 10B). In addition, the significant difference in radial diffusivity indicated poor myelin quality around mutant nerve fibres. Interestingly, the fractional anisotropy values of the mutants continued to decrease throughout the entire experiment, pointing towards a fundamentally impaired remyelination process. The poor remyelination capacity of the mutant mice was further validated by the significant decrease in luxol fast blue histochemistry of brain slices at 3 weeks after cuprizone withdrawal (Fig. 10C). In view of the previously reported increase in abundance of NG2-positive cells upon CNS demyelination, followed by return to basal levels following remyelination (Polito and Reynolds, 2005), we expected to detect an increased abundance of NG2-positive cells in the demyelinated mutant mice brains, consistent with the abnormally high abundance of these cell types in young mutant mice (Fig. 8A, left panel). Although such difference was not observed (perhaps because it occurred at an earlier time point), in contrast to the slight reduction in NG2-positive cells in mature mutant mice in the absence of cuprizone experience (Fig. 8A, right panel), a statistically significant slight increase in their abundance was in fact observed in the mutant mice at 3 weeks after cuprizone removal (Fig. 10D). GFAP staining revealed a significantly enhanced astrogliosis in the corpus callosum of the mutant compared with wild-type mice in response to cuprizone-mediated demyelination (Fig. 10E). The increase of the area parameter in the absence of increased integral optical density suggests a higher abundance of low-GFAP-expressing astrocytes.

**Discussion**

The current study represents an essential step in the understanding of eIF2B-related leucodystrophies by demonstrating that under a defined genetic background and in the absence of environmental stress, hypomorphic Eif2b5 alleles are primarily associated with
delayed and abnormal development of CNS white matter. The symptoms described in this study are mild, akin to the late-onset form in humans carrying hypomorphic Eif2b alleles with mild mutations. These include impaired motor function with involvement of the corpus callosum, internal capsule and brainstem and smaller-calibre axons, albeit without observed vanishing of the white matter (van der Knaap et al., 1998; Gallo et al., 2004; Damon-Perriere et al., 2008; Labaigue et al., 2009). The elevated abundance of oligodendrocytes in young mutant mice is in agreement with the increased number of oligodendrocytes in human patients with CACH/vanishing white matter (van der Knaap et al., 1998; Rodriguez et al., 1999; Francalanci et al., 2001; Van Haren et al., 2004). Similarly, the reduced number of astrocytes in young mutant mice is in line with their decreased abundance in human patients (Francalanci et al., 2001; Dietrich et al., 2005). Although astrocyte abundance was normalized in mature mice, this normalization was associated with a slight increase in GFAP levels (Fig. 9B, right panel), underscoring their abnormal characteristics.

Given the difference between human and mouse physiology and the mild clinical phenotype of mouse models for numerous severe human conditions, it is not surprising that the Eif2bR132H/R132H mutants suffer from neither paralysis nor early death, while the human patient homozygous for the corresponding R136H mutation died before adolescence. Nevertheless, the hypomorphic Eif2b alleles had an effect on mouse CNS white matter, despite the vast difference between human and mice white matter physiology, volume and complexity. Whereas in human patients with CACH/vanishing white matter the most severely affected white matter resides in cerebral hemispheres, the disease seems to affect other regions of the mouse brain. However, it should be noted that mice have a lower relative volume of white mater compared with humans, hence the data should not necessarily relate to the regional source of pathology but to the observation that significant portions of mouse white matter are involved. In rodents, myelination begins during the first postnatal week and peaks at the age of 3–4 weeks (Noble et al., 2005), whereas maturation of white matter pathways in
It is possible that such changes are not detected on time in development of white matter could lead to abnormal grey matter. Since grey and white matter are linked, it is expected that mal-white matter regions but also in grey matter (e.g. hippocampus). cavitating white matter disease, while MRI scans of mice brains The MRI scans of human brains indicate a progressive stage of related to the stage of the disease on diagnosis of pathology. observed differences between human and mice may also be maturation of white matter imposed by the mutated Eif2b5. The hippocampus) were observed as abnormal, reflecting the tardy regions of the mouse brain (e.g. internal capsule, brainstem and started at the age of 3 weeks, only the most slowly developing mutations have greater detrimental effects on the human brain. Since the time course MRI scans performed in the current study between humans and mice, it is conceivable that EIF2B 2005). Given the differences in duration of myelin formation be-

Figure 9 Abnormal abundance of astrocytes. Brain slices of four wild-type (wt) and four mutant (Mut) 3-week- and 4-month-old mice were immunostained using anti-GFAP antibodies followed by photography with ×25 objective and image analysis of the cortex area. (A) Representative images (scale bar = 50 μm). (B) Bars represent the average of fold change compared with the wild-type ± SEM of integral optical density (IOD) and total stained area. (C) Western blot analysis of equal amount of proteins extracted from the cortex of each brain of two 3-week-old wild-type and Mut (M) mice using antibodies specific for GFAP. Anti-β-tubulin was used for loading control.

humans continues until late childhood (Barnea-Goraly et al., 2005). Given the differences in duration of myelin formation between humans and mice, it is conceivable that Eif2b hypomorphic mutations have greater detrimental effects on the human brain. Since the course MRI scans performed in the current study started at the age of 3 weeks, only the most slowly developing regions of the mouse brain (e.g. internal capsule, brainstem and hippocampus) were observed as abnormal, reflecting the tardy maturation of white matter imposed by the mutated Eif2b5. The observed differences between human and mice may also be related to the stage of the disease on diagnosis of pathology. The MRI scans of human brains indicate a progressive stage of cavitating white matter disease, while MRI scans of mice brains may represent a more initial stage. We found changes mainly in white matter regions but also in grey matter (e.g. hippocampus). Since grey and white matter are linked, it is expected that mal-development of white matter could lead to abnormal grey matter. It is possible that such changes are not detected on time in humans and are not noted because of the overwhelming severity of the motor symptoms. Yet, it should be noted that the presentation of the late-onset form of the human disease ranges from neurologic symptoms to psychiatric manifestations or primary ovarian failure, while the typical vanishing of the white matter is sometimes absent (Labauge et al., 2009).

Delayed development of CNS white matter was apparent from the fractional anisotropy values in our MRI studies. Although the abnormally low fractional anisotropy values of young mutant mice normalized at an older age, this does not necessarily indicate that their fibre systems also normalized at this stage. While fractional anisotropy seemed to be pseudonormalized, an increase in apparent diffusion coefficient was found only at an older age (Figs 4 and 5), suggesting that this parameter is more sensitive to tissue pathology at an older age and represents a degenerative pattern. Indeed, transmission electron microscopy analysis indicated increased abundance of demyelinated axons in older mutant mice as well as axons ensheathed with split and damaged myelin, indicative of a neurodegenerative process (Fig. 6E). The abnormal content of the two major myelin proteins, myelin basic protein and PLP/DM20, at a young age, followed by normalization of myelin basic protein but not PLP/DM20 content at an older age (Fig. 7), is in agreement with abnormal myelination during development. The decreased abundance of PLP/DM20 in total hippocampus homogenates of mutant mice may result from retarded endoplasmic reticulum function, in agreement with previous findings of abnormal endoplasmic reticulum stress response in Eif2b-mutated cells (Kantor et al., 2005, 2008; van der Voorn et al., 2005; van Kollenburg et al., 2006). The involvement of PLP/DM20 proteins in the early stages of axon–oligodendrocyte interaction (Yool et al., 2001; McLaughlin et al., 2002) and their importance for normal myelination of small-calibre axons (Yool et al., 2001) and myelinated axon integrity (Griffiths et al., 1998) is consistent with the increased radial diffusivity illustrated in Fig. 6B. The age-dependent steady increase in apparent diffusion coefficient values (Fig. 5C and D) throughout the life of the mutant mice suggests a degenerative process at an older age, which progresses from the lower parts of the motor pathway. This implies a pathological condition that partially mimics the radiological presentation of CACH/vanishing white matter in human patients. Interestingly, a transgenic mice strain expressing a mild increase in PLP level exhibits late-onset demyelination and axonal degradation accompanied by loops of redundant myelin, similar to what we observed in our 65-week-old mutant mice. The neurodegenerative phenotype of the above-mentioned strain is considered to be superimposed on a mild dysmyelination (Readhead et al., 1994; Anderson et al., 1998). Given the secondary molecular effects of a complete absence of PLP/DM20 (Werner et al., 2007), it is tempting to speculate that the decreased PLP/DM20 level may also lead to abnormal molecular architecture rendering myelin sheaths more susceptible to rapid deterioration. Future experiments will provide information about myelin composition of the mutant mice and its clinical significance.

Delayed and abnormal white matter development was also apparent from the abnormal abundance of astrocytes and NG2-positive oligodendrocytes at a young age and pseudonormalization at an older age (Figs 8 and 9). NG2-expressing cells are known to occur during CNS development as part of the oligodendrocyte lineage. As the CNS matures, NG2-positive cells
differentiate and gain a more mature morphology with highly branched processes. Nonetheless, even in resting adult CNS, these cells are able to divide and revert to a simpler morphology, associated with NG2 up-regulation, in response to injury (Polito and Reynolds, 2005). The 3.4-fold increase in abundance of NG2-positive cells in 3-week-old mutant mice compared with wild-type controls and their excess proportion of processes (Fig. 8) suggests a retarded ability to differentiate further into fully mature myelinating oligodendrocytes, as appropriate, and/or a lack of proper signalling from neighbouring astrocytes. Alternatively, or additionally, there may be a compensatory proliferation of the oligodendrocyte lineage, possibly as an attempt to replace non-functional oligodendrocytes.

The lack of severe symptoms in the mutant mice may point at the importance of environmental stress in disease progression. Indeed, stress-related clinical worsening in human patients is one

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**Figure 10** Impaired recovery from cuprizone-induced demyelination. Six wild-type and six mutant (Mut) 6-week-old male mice were fed with 0.2% cuprizone-containing diet for a period of 4 weeks, followed by a normal diet for additional 4 weeks. (A) Quantitative T2 MRI analysis of the corpus callosum at different ages. Statistics were performed using Student’s t-test (one-tailed distribution with equal variance; bars represent the averages ± SEM). *P = 0.03. (B) Quantitative representation of the fractional anisotropy index, axial (λ1) and radial (λ3) diffusivities extracted from diffusion tensor imaging analysis of the corpus callosum of the wild-type and mutant groups. Statistical comparison was performed using Student’s t-test (two-tailed with unequal distribution, bars represent the averages ± SD) *P = 0.017, **P = 0.011. Note that both groups behave in a similar manner during the demyelination phase. However, recovery to normal values is observed in the wild-type group, while the fractional anisotropy and axial diffusivities of the mutant group remain at lower values. Thirteen-week-old wild-type and mutant (M) mice 3 weeks after recovery from cuprizone diet were subjected to luxol fast blue histology (C), NG2 immunohistochemistry (D) and GFAP immunohistochemistry (E). Scale bars = 500 μm, 100 μm and 50 μm, for ×2, ×10 and ×40 objectives, respectively. The marked rectangles were quantified using images taken with ×40 objective. Bars represent the average of fold change compared with the wild-type ± SEM.
of the hallmarks of Eif2b5-related white matter diseases (Schiffmann and Eloy-Stein, 2006; van der Knaap et al., 2006). Although cuprizone-mediated demyelination is not among the stress agents human patients are likely to face, this specific experimental system enabled us to provide an evidence for defective remyelination due to hypomorphic Eif2b5 alleles (Fig. 10). We speculate that a genetic tendency towards delayed and abnormal myelin formation during development may also have a similar negative effect on remyelination efficiency following sporadic brain insults. In line with this idea, we suggest that brain insults in individuals carrying hypomorphic Eif2b5 alleles result in a more rapid breakdown of abnormal myelin, followed by inefficient remyelination, imposing a major threat to demyelinated axons and leading to eventual axonal swelling and loss. The increased astrogliosis observed in the mutant mice upon demyelination (Fig. 10E) reflects abnormal performance; this is in agreement with a range of atypical astrogliosis variations previously reported for patients with CACH/vanishing white matter, depending on the specific mutation, disease severity and brain region analysed (van der Knaap et al., 1998; Rodriguez et al., 1999; Francalanci et al., 2001; Dietrich et al., 2005).

Taken together, we believe that the anomalous development of white matter in Eif2b5-mutated mice observed in our study reflects the disturbed translational regulation and coordination of a sub-class of mRNAs encoding key proteins, the identity of which remains to be uncovered. The small number and heterogeneity in the human population (genetic background, diet and environmental conditions) does not enable the detection of minor phenotypic differences. The mutant mice described in this study enabled us for the first time to reveal additional ties between Eif2b and animal growth rate, body weight and fat content (Fig. 3) as well as white matter development. Future experiments will shed light on the implications of type and timing of stress on disease onset, severity and progression. Most importantly, the mouse model will enable the identification of key mRNA transcripts whose translational control is essential for normal myelin development and maintenance.

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Supplementary material

Supplementary material is available at Brain online.

References


