Adult mouse eIF2Bε Arg191His astrocytes display a normal integrated stress response in vitro

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Vanishing white matter (VWM) is a genetic childhood white matter disorder, characterized by chronic as well as episodic, stress provoked, neurological deterioration. Treatment is unavailable and patients often die within a few years after onset. VWM is caused by recessive mutations in the eukaryotic initiation factor 2B (eIF2B). eIF2B regulates protein synthesis rates in every cell of the body. In normal cells, various types of cellular stress inhibit eIF2B activity and induce the integrated stress response (ISR). We have developed a VWM mouse model homozygous for the pathogenic Arg191His mutation in eIF2Bε (2b5ho), representative of the human disease. Neuropathological examination of VWM patient and mouse brain tissue suggests that astrocytes are primarily affected. We hypothesized that VWM astrocytes are selectively hypersensitive to ISR induction, resulting in a heightened response. We cultured astrocytes from wildtype and VWM mice and investigated the ISR in assays that measure transcriptional induction of stress genes, protein synthesis rates and cell viability. We investigated the effects of short- and long-term stress as well as stress recovery. We detected congruent results amongst the various assays and did not detect a hyperactive ISR in VWM mouse astrocytes.

Vanishing white matter (VWM) is one of the more prevalent inherited childhood brain white matter disorders1. Patients show signs of chronic as well as episodic neurological deterioration2. Episodes of fast deterioration are provoked by stresses, such as minor head trauma and febrile infections2,3. The onset of the disease varies, but is mostly observed in children with an age below 6 years4. Postmortem neuropathological examination shows selective involvement of the brain white matter, whereas grey matter structures are spared. Within the white matter, astrocytes and oligodendrocytes (together the “macroglia” or, in short, the “glia”) are selectively affected. They have abnormal morphology5,6, are immature5–8 and fail in their mature functions, leading to a profound lack of myelin (oligodendrocyte function) and to deficient scar tissue formation (astrocyte function)5,9.

VWM patients have bi-allelic recessive mutations in any of the five genes encoding the subunits of the eukaryotic translation factor 2B (eIF2B)10,11. We recently developed a VWM mouse model with a homozygous Arg191His mutation in eIF2Bε (2b5ho), representing the human Arg195His mutation12. This founder mutation in the Cree population in North America13 causes a severe variant of VWM14. The “2b5ho mouse model” recapitulates the human disease, with neurological dysfunction as well as astrocyte and oligodendrocyte abnormalities, similar to those observed in patients12. Astrocyte dysfunction is most likely the primary one with oligodendrocyte dysfunction being secondary12.

eIF2B functions as guanine nucleotide exchange factor (GEF), exchanging GDP for GTP on its trimeric substrate eIF215. eIF2-GTP binds a charged initiator methionyl-transfer RNA constituting the ternary complex. This complex binds to the small ribosomal subunit, which binds to and scans the 5′untranslated region. Upon start codon recognition and delivery of the methionyl-tRNA, eIF2-GTP is hydrolyzed to inactive eIF2-GDP, which leaves the ribosome. eIF2B reactivates eIF2-GDP to eIF2-GTP; a new ternary complex can form and initiate a next round of translation. In this whole process, eIF2B activity is a key regulator, determining the translation rate,
especially under proteotoxic stress conditions. Regulation of eIF2α activity occurs in several ways. Most studied is the regulation by phosphorylation of serine 51 (Ser51) of the eIF2α subunit, which leads to activation of the integrated stress response (ISR). Four kinases are known that phosphorylate Ser51 upon specific triggers associated with proteotoxicity: protein kinase activated by double-stranded RNA (PKR), PKR-like endoplasmic reticulum kinase (PERK), general control non-derpressible 2 (GCN2) and heme-regulated inhibitor (HRI). PERK is activated by unfolded proteins. PKR is activated by accumulation of double-stranded RNA during infection. GCN2 is activated by uncharged tRNAs when amino acids are low. HRI is activated during heme deprivation and oxidative stress. eIF2α phosphorylated at Ser51 binds tightly to eIF2B and acts as a competitive inhibitor. Consequently, general protein synthesis is decreased. Translation of specific proteins is increased upon eIF2α phosphorylation, with activating transcription factor 4 (ATF4) as a prototypic example. ATF4 induces the transcription of genes encoding proteins such as CHOP homologous protein (CHOP), growth arrest and DNA damage-inducible protein 34 (GADD34) and Tribbles homolog 3 (TRIB3).

Previously we reported that VWM patients’ brains show activation of the unfolded protein response (UPR) in glia. The UPR is activated upon endoplasmic reticulum (ER) stress, which activates three independent sensors in the ER membrane: PERK, activating transcription factor 6 (ATF6) and Inositol-requiring enzyme 1α (IRE1α). Upon ER stress, PERK dimerizes, autophosphorylates and promotes the ISR. In addition, ATF6 leaves the ER and migrates towards the Golgi where it is processed into ATF6-c, an active transcription factor that induces expression of the gene encoding protein disulfide isomerase family A member 4 (Pdia4) and other genes. Furthermore, IRE1α dimerizes, autophosphorylates and induces the synthesis of the transcription factor X-box-binding protein 1 s (XBP1s) through a cytoplasmic splicing event of the unspliced mRNA precursor XBP1u. Each transcription factor selectively induces transcription of effector genes that allow cells to cope with the stressor, helping to restore ER function to normal or reduce apoptosis if the stressor is too severe.

We and others have previously hypothesized that eIF2B mutations lead to hypersensitivity of the ISR and have investigated this hypothesis in patients’ lymphoblasts and fibroblasts with thapsigargin or heat shock as stress inducers for the ISR. Others have addressed the hypothesis by studying UPR or ISR induction in rat and human oligodendrocyte cell lines or hamster ovary cell lines. We and others have previously hypothesized that eIF2B mutations lead to hypersensitivity of the ISR and have investigated this hypothesis in patients’ lymphoblasts and fibroblasts with thapsigargin or heat shock as stress inducers for the ISR. Others have addressed the hypothesis by studying UPR or ISR induction in rat and human oligodendrocyte cell lines or hamster ovary cell lines. We and others have previously hypothesized that eIF2B mutations lead to hypersensitivity of the ISR and have investigated this hypothesis in patients’ lymphoblasts and fibroblasts with thapsigargin or heat shock as stress inducers for the ISR. Others have addressed the hypothesis by studying UPR or ISR induction in rat and human oligodendrocyte cell lines or hamster ovary cell lines.

Results

Wt and 2b5ho astrocytes respond similarly to short term UPR or ISR induction. To assess whether 2b5ho primary astrocytes are hypersensitive to ISR activation, we cultured astrocytes in the presence of thapsigargin or tunicamycin. These compounds lead to inhibition of eIF2B activity via phosphorylation of eIF2α at Ser51 by PERK (ISR-activating kinase) and activate the ATF6 and IRE1α branches of the UPR. We subjected wt and 2b5ho astrocytes to either compound for a short interval (4 h). We assessed UPR activation by quantifying Trib3, Xbp1s and Pdia4 mRNAs that are specifically induced by transcription factors ATF4, Xbp1s and ATF6-c respectively. The induction of Trib3, Xbp1s and Pdia4 expression was substantial and significant but did not differ between wt and 2b5ho cells (Fig. 1). One could argue that tunicamycin and thapsigargin are not suitable for assessing the ISR in cells with mutated eIF2B. Both compounds activate the ATF6 and IRE1α branches of the UPR, each leading to activation of fairly distinct transcription programs. It cannot formally be excluded that crosstalk between the three branches might mask an abnormal response to eIF2α phosphorylation in 2b5ho astrocytes. To further address this issue, we investigated selective activation of the ISR in 2b5ho astrocytes using four additional compounds (CCT020312, BEPP, B’dCPU, halofuginone) that were recently described as ISR inducers. We subjected astrocytes to these compounds and measured induction of UPR mRNA markers. Activation of the ATF6 and IRE1α branches of the UPR was not observed as judged from changes in Xbp1s + u/a and Pdia4 (Fig. 1). Treatment with either of these compounds induced the ISR to differing degrees as judged from the induction of Trib3 mRNA, which ranged from 2-fold for B’dCPU to 15-fold for halofuginone (Fig. 1). Thapsigargin and tunicamycin induced Trib3 mRNA expression approximately 20-fold (Fig. 1). Yet the ATF4-induced transcription did not differ between wt and 2b5ho (Fig. 1). We chose Trib3 mRNA induction as a robust and sensitive marker for the integrated stress response as assessment of Ddit3 mRNA expression was less sensitive and ATF4 protein induction more variable in our hands (Supplementary Fig. S1 and Supplementary Fig. S2). Still, differences between wt and 2b5ho astrocytes were not observed for any of the stress markers (Fig. 1, Supplementary Fig. S1 and Supplementary Fig. S2). These data suggest that activation of neither the ISR nor UPR was significantly affected by the Arg191His mutation in eIF2Be.

We next investigated the inhibition of protein synthesis in response to ISR. We subjected wt and 2b5ho astrocytes to either of the six ISR-inducing compounds and measured protein synthesis for a two-hour pulse using an azidohomoalanine (AHA) incorporation assay (Fig. 2). This sensitive and robust protocol relies on replacing the amino acid methionine in the culture medium with its analogue AHA. AHA-incorporation was measured...
Figure 1. The UPR and ISR markers are similarly induced in wt and 2b5ho astrocytes upon short-term stress induction. UPR was induced by ER stressors tunicamycin (TM) or thapsigargin (TG). ISR was induced by treating cells with CCT020312 (CCT), BEPP, BTdCPU (BT) or halofuginone (HF) for 4 h. The kinases activated by ISR-inducing compounds are indicated (PERK, PKR, HRI or GCN2). The relative expression of mRNA markers of the UPR, Trib3, Xbp1s+u/u and Pdia4, was measured by qPCR after treatment. Values are fold change relative to vehicle-treated wt astrocytes. Graphs show average ± SD (n = 3). P-values are shown in supplementary Table 1. Trib3, Xbp1s + u/u and Pdia4 are significantly increased by tunicamycin and thapsigargin while only Trib3 is significantly increased by CCT020312, BEPP, BTdCPU or halofuginone indicating ISR specificity. The increase is similar for wt and 2b5ho astrocyte cultures.
for 2 h in the presence or absence of tunicamycin or thapsigargin. Each treatment inhibited protein synthesis by approximately 50%, indicating that protein synthesis in wt and 2b5 ho astrocytes was inhibited similarly (Fig. 2).

The induction of the ISR by tunicamycin and thapsigargin was quite pronounced and perhaps too strong to detect possibly subtle differences in 2b5 ho astrocytes. We thus repeated the experiment with a lower concentration of thapsigargin (0.33 μM) (Fig. 2). Protein synthesis was still significantly inhibited, but less than observed with previous concentration of thapsigargin (1 μM), confirming that the ISR activation by 0.33 μM thapsigargin was not maximal. The ISR-inducing compounds CCT020312 and halofuginone each inhibited protein synthesis by approximately 50%, similar to the inhibition observed with tunicamycin or thapsigargin (Fig. 2). The inhibition of protein synthesis was relatively small for BTdCPU and was not significant for BEPP (Fig. 2). Again, we did not observe a significant difference in response between wt and 2b5 ho astrocytes.

Wt and 2b5 ho astrocytes respond similarly to long term UPR induction and recovery. So far we have investigated ISR and UPR induction in primary astrocytes over a relatively short interval. Possibly, 2b5 ho primary astrocytes show differences in ISR activation when the exposure to stress is extended. This proposed effect would have been missed in the previous experiments. We extended the exposure to the selected stressors to 16 h and assessed mRNA expression (Fig. 3 and Supplementary Fig. S3). Both treatments induced significant UPR mRNA expression (Supplementary Table 1). We did not find consistent differences in stress response between wt and 2b5 ho astrocytes. The response in 2b5 ho astrocytes appeared to be normal at both short and long times.

Alternatively, 2b5 ho astrocytes may recover abnormally from stress. We further increased the stress duration to 24 h with thapsigargin in wt and 2b5 ho astrocytes. We then replaced the culture medium and omitted thapsigargin. The cells were then left to recover for 24 h or 72 h. The UPR marker mRNAs of all three branches decreased over time during stress recovery (Fig. 4, Supplementary Fig. S3). Trib3, Ddit3, Xbp1s + u, Xbp1u and Pdia4 mRNA expression were similar in wt and 2b5 ho astrocytes at all measured time points. Together these results indicate that the induction of the UPR or subsequent recovery from it do not differ between wt and 2b5 ho astrocytes at the mRNA level (Fig. 4).

Wt and 2b5 ho astrocytes respond to ISR and UPR induction at the level of cell viability. After investigation of the ISR and UPR on mRNA as well as protein synthesis level, we interrogated the cellular effects in response to stress. We assessed viability of both wt and 2b5 ho astrocytes in response to long term exposure to the various compounds (i.e. 24 h and 96 h) as well as recovery (24 h stressor and subsequent 72 h recovery). None of the compounds significantly reduced cell viability at 24 h with the exception of CCT020312 (Fig. 5). After 96 h cell viability was compromised by PERK activators tunicamycin, thapsigargin and CCT020312, but not by
the ISR activators that mediate the response via PKR, GCN2 or HRI. To assess stress recovery the stressor was removed after 24 h and cell viability was determined after 96 h. The PERK activators negatively impacted on cell viability under all conditions, whereas the other compounds did not. Still, the cell viability was similar between wt and 2b5ho astrocytes under all tested conditions. As CCT020312 already strongly reduced cell viability at 24 h (approximately 50%), we tested cell viability at 4 h and found that also at this early time-point it was reduced by approximately 40% (Fig. 6). Again, this assay did not detect a different cellular response to UPR or ISR induction in 2b5ho astrocytes, in line with the results obtained thus far. The reduction in cell viability varied between the different stressors but, importantly, was similar for wt and 2b5ho astrocyte cultures.

Figure 3. The UPR markers are similarly induced in wt and 2b5ho astrocytes upon long-term stress induction. UPR was induced by ER stressors tunicamycin (TM) or thapsigargin (TG) for 16 h. The relative expression of UPR mRNA markers Trib3, Xbp1s +/u and Pdia4 was measured by qPCR. Values are fold change relative to vehicle-treated wt astrocytes. Graphs show average + SD (n = 3). P-values are shown in supplementary Table 1. All markers are significantly increased after tunicamycin or thapsigargin treatment. The increase is similar for wt and 2b5ho astrocyte cultures.

Figure 4. The UPR recovers similarly in wt and 2b5ho astrocytes. UPR was induced by ER stressor thapsigargin (TG) for 24 h. TG was subsequently removed and cells were left to recover for 24 (+TG + 24 h rec) or 72 h (+TG + 72 h rec). The relative expression of UPR mRNA markers Trib3, Xbp1s +/u and Pdia4 was measured at indicated times by qPCR. Values are the fold change relative to vehicle-treated wt astrocytes (−TG, 24 h). Graphs show average + SD (n = 3). P-values are shown in supplementary Table 1. Trib3, Xbp1s +/u and Pdia4 are is significantly increased at all time points. The increase is similar for wt and 2b5ho astrocyte cultures.
Figure 5. Cell viability upon UPR or ISR induction is not differentially affected in wt and 2b5\textsuperscript{ho} astrocytes. UPR was induced by ER stressors tunicamycin (TM) or thapsigargin (TG). ISR was induced by CCT020312 (CCT), BEPP, BTdCPU (BT), or halofuginone (HF). The kinases activated by ISR compounds are indicated (PERK, PKR, HRI or GCN2). Cell viability was measured 24 and 96 h after stress induction without and with recovery after 24 h. Values are relative cell viability compared to vehicle-treated cultures. Graphs show average ± SD (n = 3; CCT in wt, n = 2). P-values are shown in supplementary Table 1. Tunicamycin and CCT020312 significantly reduces cell viability under all test conditions. BEPP only significantly reduces the cell viability after 24 h while thapsigargin significantly reduced cell viability only after 96 h. The cell viability was similar for wt and 2b5\textsuperscript{ho} astrocyte cultures.
the ATF6- and XBP1s-driven transcription programs, confirming that they do not activate a full UPR (Fig. 1). 

or CCT020312, suggesting a relatively high activity. Also, the ISR activators tested did not significantly activate activity. The latter could be true for PERK, which is activated efficiently in response to thapsigargin, tunicamycin eIF2 kinases. Alternatively, the specific kinases in mouse primary astrocytes may differ in expression or specific taken up by cells with differing efficiency or if they differ in their effectiveness in activating the relevant specific is an interesting observation. However, at the moment we cannot discriminate whether these compounds are 

Together our data indicate that under low, high, long, short UPR or ISR induction an abnormal response in 

2b5 ho which is known to be disturbed in VWM 35. The eIF2B 

dehomoygous for the equivalent Arg191His mutation is also intrinsically affected in eIF2B activity.

We now report that the 2b5 ho astrocytes do not show signs of expected consequences of reduced eIF2B activity: they grew with similar rates and incorporated the AHA label with similar efficiency as wt astrocytes, indicative of normal protein synthesis rates (Fig. 2A). Surprisingly, the ISR and UPR in astrocytes were not significantly affected by the Arg191His mutation in eIF2Bε. Together these observations suggest that in primary astrocyte 

tive phenotype in patients14. Recombinant human eIF2B with the Arg195His mutation in eIF2Bε showed a significantly decreased enzymatic activity to approximately 50% of wt eIF2B activity42, indicating that the 2b5 ho mouse model homozygous for the equivalent Arg191His mutation is also intrinsically affected in eIF2B activity.

We previously found that astrocytes are likely the cells that are primarily affected cells in VWM12. At the start of the study, we selected astrocyte cultures for testing ISR or UPR hypersensitivity, as this system allows multiple manipulations to modulate eIF2B activity. We focused on the 2b5 ho astrocytes as this mouse model showed a more severe phenotype than the 2b4 ho model, which is homozygous for the eIF2Bδ mutation Arg484Trp12. The 2b5 ho astrocytes have recently been reported to reveal differences in metabolite accumulation and in synthesis rates for 80 proteins. The function of some of these eIF2Bε,Arg191His-regulated proteins is linked to astrocyte differentiation, which is known to be disturbed in VWM35. The eIF2Bε Arg195His mutation leads to a severe neurological phenotype in patients14. Recombinant human eIF2B with the Arg195His mutation in eIF2Bε showed a significantly decreased enzymatic activity more than 50%33. Alternatively, it is possible that reduced eIF2B activity in astrocytes derived from embryonic mice45. BEPP grossly reduced the viability of mouse embryonic fibroblast after 72 h, while halofuginone did not affect the viability of human fibroblasts or osteosarcoma cells37,46,47. These 

Discussion

We previously found that astrocytes are likely the cells that are primarily affected cells in VWM12. At the start of the study, we selected astrocyte cultures for testing ISR or UPR hypersensitivity, as this system allows multiple manipulations to modulate eIF2B activity. We focused on the 2b5 ho astrocytes as this mouse model showed a more severe phenotype than the 2b4 ho model, which is homozygous for the eIF2Bδ mutation Arg484Trp12. The 2b5 ho astrocytes have recently been reported to reveal differences in metabolite accumulation and in synthesis rates for 80 proteins. The function of some of these eIF2Bε,Arg191His-regulated proteins is linked to astrocyte differentiation, which is known to be disturbed in VWM35. The eIF2Bε Arg195His mutation leads to a severe neurological phenotype in patients14. Recombinant human eIF2B with the Arg195His mutation in eIF2Bε showed a significantly decreased enzymatic activity to approximately 50% of wt eIF2B activity42, indicating that the 2b5 ho mouse model homozygous for the equivalent Arg191His mutation is also intrinsically affected in eIF2B activity.

Figure 6. Cell viability upon short-term CCT020312 treatment is not differentially affected between wt and 2b5 ho astrocytes. Cell viability was measured after 4 h of CCT020312 (CCT) treatment. Values are relative cell viability compared to vehicle-treated cultures. Graphs show average ± SD (n = 3, wt CCT n = 2). P-values are shown in supplementary Table 1. Although cell viability was significantly reduced, no significant difference in response was observed between wt and 2b5 ho astrocyte cultures.

In general, viability of the mouse astrocyte cultures was not markedly affected by the stress inducers, and no differences were noted between wt and mutant cells. With the exception of CCT020312, none of the compounds greatly reduced astrocyte viability after exposure interval of 24 h. Other cell types such as neuronal cell lines or primary rat cardiac myocytes show a marked reduction in cell viability upon 24 h thapsigargin or tunicamycin treatment33,44. In support of our findings UPR activation by 24 h thapsigargin did not lead to reduced cell viability in astrocytes derived from embryonic mice45. BEPP grossly reduced the viability of mouse embryonic fibroblast after 72 h, while halofuginone did not affect the viability of human fibroblasts or osteosarcoma cells37,46,47. These
results suggest that the cell type and growth conditions highly influence the response to UPR or ISR activation. CCT020312 was initially identified in a screen searching for a cell cycle arrest factor and was subsequently found to activate PERK as underlying mechanism. This is an interesting finding: activation of PERK reduced cell viability most in adult murine astrocytes compared to the other stressors. However, under these circumstances no differences were observed between wt and Eif2b5Δ primary astrocytes.

In VWM patients' brains the ISR/UPR is activated in glia. It is not known whether the activated UPR in patients' glia is a hallmark of a protective reaction to the disease or if it contributes to neuropathology or clinical signs in patients. Patients show periods of rapid deterioration as a consequence of physical stresses. Possibly, during these events the ISR or UPR in glia become hyperactivated as a consequence of the eIF2B mutations. We cannot rule out that a minor difference in stress responses in 2b5Δ primary astrocytes was missed in the current study, despite the severity of the eIF2Bε Arg191His mutation in patients. Nonetheless, the differences in ATF4 and CHOP protein expression were readily detected in VWM patients' brain tissue by qPCR and Western blotting and it is reasonable to conclude that these differences in expression were not recapitulated in the current cell system. On the basis of the current findings, we cautiously propose that the eIF2Bε Arg191His mutation does not intrinsically affect the kinetics of the ISR or UPR in cultured astrocytes.

Probably, the mutant mouse astrocyte cultures are not representative of the astrocytes in patients' or mutant mouse brains. Both astrocyte morphology and Gfapε expression have been reported to be abnormal in patient and 2b5Δo mouse brain. These features are not recapitulated in the 2b5Δo astrocyte cultures. We should point out that 2b5Δo primary astrocytes from E18 mice were deficient in supporting oligodendrocyte maturation in vitro, despite similar morphology and GFAP-expression when compared to wt astrocytes. Human astrocyte cultures from VWM patients are a very limited resource and therefore we cannot address whether human astrocytes would be a superior model to the murine astrocytes. We suspect that fully recapitulating the phenotype of cells in intact tissue. Responses to febrile stress or tissue damage in patients' brain may also involve ISR- or UPR-independent mechanisms that are not yet fully understood. The elevation of ISR/UPR markers in VWM brain may reflect the interplay between additional factors, including ones that are not intrinsic to astrocytes.

Materials and Methods

Animals. All experiments were carried out under the Dutch/European law and with approval of the local animal care and use committee of the VU University of Amsterdam (permit number FGA 11-05, FGA 14-04). Wt and 2b5Δo mice were used. All animals were weaned at P21 and subsequently had ad libitum access to food and water. The mice were housed with a 12 h light/dark cycle. Mice were genotyped as described.

Astrocyte culture. Astrocytes were isolated from gender-matched 4-month-old wt and 2b5Δo mice in parallel. Mice were sacrificed by cervical dislocation. Brains were taken out; the olfactory bulb, cerebellum and cortex were removed. Astrocytes were isolated from the remaining structures, as described. The newly synthesized proteins were cultured with the indicated compounds for the indicated time. Cells were stressed with various compounds for indicated times. At the start of treatment, we replaced the culture medium with DMEM/P12 medium supplemented with UPR activators tunicamycin (2.5 µg/ml; Sigma) and thapsigargin (0.33 and 1 µM; Sigma) or GCN2 activator halofuginone (10 nM; Cayman Chemical Company) or HRI activator BtDCPU (KM097485C) (6 µM; Thermofisher) or PKR activator BEPP (10 µM; Sigma) or PERK activator CCT020312 (10µM; Millipore) which specifically activate the ISR. All compounds were dissolved in DMSO. Control cultures were simultaneously treated with equal amounts of DMSO (vehicle control).

RNA isolation, cDNA synthesis and qPCR. Astrocytes were plated in 10 cm dishes (~750,000 cells/dish). The astrocytes were cultured until 80% confluent. Cells were subsequently treated with the indicated stressors for the indicated time. Cells were washed with cold PBS (Gibco) and collected in TRIzol™ Reagent (Invitrogen). RNA was isolated as described. RNA quality and quantity was determined by measuring the A260 and A280 (Nanodrop 2000, Thermo Scientific). cDNA was synthesized and mRNA levels were measured as described. For each 10 µl qPCR sample a mixture of SYBR green (Roche), primers (1 pmol/µl) and cDNA (0.1 µl) was used. The primers used were validated (Supplementary Fig. S4) and are listed in Table 1. Gapdh mRNA was used as reference for normalization. Sequence analyses were performed on cDNA from 42 samples from wt and 2b5Δo replicated in independent cultures derived from different mice (number of experiments is indicated in figure legend as e.g. n = 3).

Determination of protein synthesis rates. Astrocytes were plated in 6 cm dishes (~250,000 cells/ dish) and were cultured until 80–90% confluency. Indicated stressors were added for 4 h. Protein synthesis was determined with AHA incorporation, as described. AHA (Bachem) was added for the last two hours of the experiment to allow sufficient AHA incorporation into newly synthesized proteins. The newly synthesized proteins were quantified visualized as described. The amount of staining is corrected for the total protein load determined by Gel Doc™ EZ System (Biorad).

Cell viability. Astrocytes were plated in 1/2 area 96 well plates (~5,000 cells/well; Greiner Bio–one). Cells were cultured with the indicated compounds for the indicated time. Cell viability was measured based on the quantification of intracellular ATP levels as a measure for metabolically active cells. The assay is performed according
Table 1. Oligonucleotide primers.

| Gene name | Forward (5’ → 3’) | Reverse (5’ → 3’) |
|-----------|-------------------|------------------|
| Gapdh     | GTGCTGATATGTCGTTGAG | TCGTGGTCACACCCATCAC |
| Ddit3     | CGTTGATATGTCGTTGAG | TGTTCTCCTCCTTCGTTTCC |
| Trib      | TGTCCCTCAAGACATTGTGAAGGAGAAGAG | GTAGGATGCCCGAGGACTGAGATC |
| Xbp1u     | CGACGACTCAAGACTATGTG | CCAACCTTGCCAGAATGGC |
| Xbp1s+u   | TCCCGGACGGGGTCCGACG | CCAACCTTGCCAGAATGGC |
| Pha4      | GGTATCATGCGGGCTCCTTCAG | GGAGACTGCTATTTCAGG |

Statistical analysis. The software program Factor was used to correct for differences between experiments but not between other conditions within experiments (genotype, treatments)\(^{30}\). For the qPCR experiments, all data points in one experiment (displayed in one graph) are divided by the average of data point for each mRNA in all wt DMSO. For the AHA incorporation experiments the average incorporation of all wt DMSO was set at 100%. The cell viability values depended heavily on cell number and therefore these experiments were normalized separately for wt and 2b\(^5\)o. Statistical differences were determined using SPSS. We tested if the effect of treatment was different between wt and 2b\(^5\)o astrocytes (univariate analysis of variance, UNIANOVA, two-tailed).

Data availability. The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

References
1. van der Knaap, M. S., Breiter, S. N., Naidu, S., Hart, A. A. & Valk, J. Defining and categorizing leukoencephalopathies of unknown origin: MR imaging approach. Radiology 213, 121–133, https://doi.org/10.1148/radiology.213.1.r99se121 (1999).
2. van der Knaap, M. S. et al. A new leukoencephalopathy with vanishing white matter. Neurology 48, 845–855 (1997).
3. Fogli, A. et al. The effect of genotype on the natural history of eIF2β-related leukodystrophies. Neurology 62, 1509–1517 (2004).
4. van der Knaap, M. S., Pronk, J. C. & Schepers, G. C. Vanishing white matter disease. Lancet Neurol 5, 413–423, https://doi.org/10.1016/S1474-4422(06)70440-9 (2006).
5. Bugiani, M. et al. Defective glial maturation in vanishing white matter disease. J Neuropath Exp Neurol 70, 69–82, https://doi.org/10.1097/NEN.0b013e318203ae74 (2011).
6. Van Haren, K., van der Voorn, J. P., van der Knaap, M. S. & Powers, J. M. The death and life of oligodendrocytes in vanishing white matter disease. J Neurophysiol 83, 618–630 (2000).
7. Middendorf, J. et al. GFAP delta in radial glia and subventricular zone progenitors in the developing human cortex. Development 137, 313–321, https://doi.org/10.1242/dev.014630 (2010).
8. Raponi, E. et al. S100B expression defines a state in which GFAP-expressing cells lose their neural stem cell potential and acquire a more immature developmental stage. Glia 55, 165–177, https://doi.org/10.1002/glia.20445 (2007).
9. Dietrich, J. et al. EIF2β mutations compromise GFAP + astrocyte generation in vanishing white matter leukodystrophy. Nat Med 11, 277–283, https://doi.org/10.1038/nm1195 (2005).
10. Leegwater, P. A. et al. Subunits of the translation initiation factor eIF2β are mutant in leukoencephalopathy with vanishing white matter. Nat Genet 29, 383–388, https://doi.org/10.1038/ng764 (2001).
11. van der Knaap, M. S. et al. Mutations in each of the five subunits of translation initiation factor eIF2β can cause leukoencephalopathy with vanishing white matter. Ann Neurol 51, 264–270 (2002).
12. Dooves, S. et al. Astrocytes are central in the pathomechanisms of vanishing white matter. J Clin Invest 126, 1512–1524, https://doi.org/10.1172/JCI83908 (2016).
13. Fogli, A. et al. Cree leukencephalopathy and CACH/VWM disease are allelic at the EIF2B5 locus. Ann Neurol 52, 506–510, https://doi.org/10.1002/ana.10339 (2002).
14. Black, D. N. et al. Encephalitis among Cree children in northern Quebec. Ann Neurol 24, 483–489, https://doi.org/10.1002/ana.4102404042 (1988).
15. Proust, C. G. Regulation of eukaryotic initiation factor eIF2β. Prog Mol Cell Biol 26, 95–114 (2001).
16. Tanouchi, S., Miyake, M., Tsugawa, K., Oyadomari, M. & Oyadomari, S. Integrated stress response of vertebrates is regulated by four eIF2β kinases. Sci Rep 6, 32886, https://doi.org/10.1038/srep32886 (2016).
17. Pakos-Zebrucka, K. et al. The integrated stress response. EMBO Rep 17, 1374–1395, https://doi.org/10.15252/embr.201642195 (2016).
18. Harding, H. P. et al. Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol Cell 6, 1099–1108 (2000).
19. Proust, C. G. eIF2 and the control of cell physiology. Semin Cell Dev Biol 16, 3–12, https://doi.org/10.1016/j.semcdb.2004.11.004 (2005).
20. Han, J. et al. ER-stress-induced transcriptional regulation increases protein synthesis leading to cell death. Nat Cell Biol 15, 481–490, https://doi.org/10.1038/nclb2738 (2013).
21. Novoa, I., Zeng, H., Harding, H. P. & Ron, D. Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2β. J Cell Biol 153, 1011–1022 (2001).
22. Ohoka, N., Yoshihi, S., Hattori, T., Onozaki, K. & Hayashi, H. TRB3, a novel ER stress-inducible gene, is induced via ATF4–CHOP pathway and is involved in cell death. EMBO J 24, 1243–1255, https://doi.org/10.1038/emboj.2005096 (2005).
23. van Kollenburg, B. et al. Glia-specific activation of all pathways of the unfolded protein response in vanishing white matter disease. J Neuropath Exp Neurol 65, 707–715, https://doi.org/10.1097/01.jn.0000228102.72539.50 (2006).
24. van der Voorn, J. P. et al. The unfolded protein response in vanishing white matter disease. J Neuropath Exp Neurol 64, 770–775 (2005).
25. Wu, J. et al. ATF6βalpha optimizes long-term endoplasmic reticulum function to protect cells from chronic stress. Dev Cell 13, 351–364, https://doi.org/10.1016/j.devcel.2007.07.005 (2007).
26. Lee, A. H., Iwakoshi, N. N. & Glimcher, L. H. XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. Mol Cell Biol 23, 7448–7459 (2003).
27. Walter, P. & Ron, D. The unfolded protein response: from stress pathway to homeostatic regulation. Science 334, 1081–1086, https://doi.org/10.1126/science.1209038 (2011).
47. Lamora, A. & Perry, D. C. Dantrolene is cytoprotective in two models of neuronal cell death.

40. Stockwell, S. R.

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Additional Information

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